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Pulmonary Delivery of Engineered Exosomes to Suppress Postoperative Melanoma Lung Metastasis through Preventing Premetastatic Niche Formation

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Abstract

Premetastatic niche (PMN) is a prerequisite for initiation of tumor metastasis. Targeting prevention of PMN formation in distant organs is becoming a promising strategy to suppress metastasis of primary tumor. Based on “organotropic metastasis”, melanoma tends to metastasize to lungs, where granulocytic myeloid-derived suppressor cells (G-MDSCs) recruitment in lungs significantly contributes to the PMN formation. Herein, functional exosomes (GExoI) were designed to present pulmonary targeting peptide GFE1 on the membrane and load PI3Kγ inhibitor (IPI549) inside, aiming at suppressing postoperative lung metastasis of melanoma. In postoperative mice model, intravenously injected GExoI could significantly accumulate in lungs and release IPI549 to block G-MDSCs recruitment through interfering with CXCLs/CXCR2/PI3Kγ signaling. The increased percentages of CD4+ T cells and CD8+ T cells in lungs could transform microenvironment from immunosuppression to immunostimulation, leading to metastasis inhibition. This study suggests an effective anti-metastasis strategy of targeting prevention of PMN formation through specifically blocking G-MDSCs recruitment.
**Key words:** Premetastatic niche, Exosome, Pulmonary delivery, Anti-metastasis, IPI549

**Introduction**

Cancer has become the leading cause of threatening human's health, and various traditional treatments including surgery, radiotherapy and chemotherapy have been applied to treat cancer [1]. However, due to metastasis, the treatment failure and mortality occurs in more than 90% of individuals with malignant tumors [2-4]. Various anti-metastasis strategies have been developed to improve the treatment of cancer, which mainly focus on the dynamic plasticity of tumor cells, such as the inhibition of migration, adhesion and angiogenesis of tumor cells by drugs[5]. However, as the significant resistance of metastatic tumors to chemotherapy and the limited delivery efficiency of drugs to the tumor metastasis microenvironment, the current treatment of metastasis lacks effectiveness [6]. Moreover, surgery as a necessary step in the treatment of solid cancers, may also facilitate the metastatic process by dissemination of tumor cells during the surgical procedure, local and systemic release of growth factors, and cellular immune suppression[7]. It has been well established that organs of future metastasis are not passive receivers of circulating tumor cells, and before metastasis, the microenvironment of the target organs has been altered, such as marrow derived cell aggregation, continuous inflammation and oxygen free radical increase, which is beneficial for creating a suitable niche for metastatic tumor cells colonization [8, 9]. This metastasis-favorable microenvironment created in the distant organs by the primary tumor is defined as the premetastatic niche (PMN) [10-12], which is modified by primary tumor-derived factors and extracellular vesicles, bone marrow-derived cells, stromal cells and the cytokines they produce [13]. Therefore, preventing the PMN formation probably is an effective strategy to inhibit the occurrence of metastasis. However, it is challenging to specifically destroy the PMN formation in organs of future metastasis, and only limited approaches have been exploited [14, 15].

Granulocytic myeloid-derived suppressor cells (G-MDSCs) are a major population of immature myeloid cells that accumulate in patients with cancer and appear in the early PMN [16-19]. These G-MDSCs are able to induce immunosuppression, increase vascular permeability and promote the colonization and metastatic growth of disseminated tumor cells in PMN [20-22]. The CXCLs/CXCR2 signaling axis plays a vital role in prompting G-MDSCs to enter tissues from bone marrow [23-25]. It has been reported that the primary tumor can recruit CXCR2-positive G-MDSCs by elevating CXCL1
in premetastatic liver tissue to form a PMN [26], which suggests that the destruction of
CXCLs/CXCR2 signaling axis in G-MDSCs is a potential approach to prevent PMN formation and
inhibit the metastasis. Phosphoinositide 3-kinase gamma (PI3Kγ) pertains to a signal transducing
enzyme that plays an important role in CXCLs/CXCR2 signaling axis. Thus, PI3K-γ inhibition in
G-MDSCs may be effective on blocking G-MDSCs recruitment for PMN elimination. However, since
PI3K-γ is also a critical determinant of the proportion of neutrophil chemotaxis [27], non-specific
organs, resulting in severe immune system defects in the body. Therefore, realization of specific PI3K-γ
inhibition in G-MDSCs will become a safe and feasible approach for inhibiting PMN formation and
further preventing the metastasis.

Clinical observations suggest that most cancers usually can metastasize to specific organs, so called
“organotropic metastasis”. For examples, melanoma tends to metastasize to lungs; colorectal cancer
tends to metastasize to livers; prostate cancers selectively metastasize to bone [28-30]. Such
phenotypes offer opportunities to prevent tumor metastasis by specific blocking PMN formation in the
organs of future metastasis. Despite the promising anti-metastasis potential of blocking PMN formation,
its clinical application is restricted by the lack of organ specificity after systemic administration.
Development of biocompatible and organ-targeting drug delivery system will be required. Exosomes
naturally secreted by various cell types is membrane-enclosed vesicles with sizes of 30–200 nm [31].
Those exosomes obtained from the patient’s own cells have desirable biocompatibility, low
immunogenicity and prolonged systemic circulating ability [32-34]. While various exosomes have been
used as carriers for anti-tumor metastasis therapy, the affinity of exosomes to mother cells is not
sufficient for exosomes to specifically aggregate in one organ of the body [35-37]. Current methods
which endow exosomes with the specific targeting ability involve genetic manipulation of the producer
cells or chemical conjugation of peptides to the surface of exosomes [32, 38-41]. Therefore, based on
above methods, organ-specific targeting exosomes can be obtained with organ-targeting peptides.
Furthermore, the engineering organ-targeted exosomes can site-specifically deliver drugs to specific
metastasis site, potentially directionally inhibiting the PMN formation in organs of premetastasis.
Figure 1 Schematic illustration for anti-tumor metastatic effects of \( \text{Exo}^{l} \). a Preparation of \( \text{Exo}^{l} \) from MSCs that were transfected with the recombinant plasmid encoding Lamp2b-GFE1 fusion protein and incubated with the PI3K\( \gamma \) inhibitor, IPI549. b Process of pulmonary delivery of \( \text{Exo}^{l} \) through GFE1 binding to the surface of pulmonary vascular endothelial cells and prevention of PMN formation through IPI549-mediated blockage of G-MDSCs recruitment. The decreased percentages of monocytic myeloid-derived suppressor cells (mo-MDSCs) and M2 phenotype macrophage and the increased percentages of CD4\( ^{+} \) T cells and CD8\( ^{+} \) T cells in lungs could transform microenvironment from immunosuppression to immunostimulation, blocking the lung metastasis of melanoma.

In the present study, functional exosomes were engineered for targeted delivery of the PI3K\( \gamma \)
inhibitor (IPI549) to lung of a melanoma mouse model, aiming at preventing the PMN formation and
suppressing postoperative lung metastasis of melanoma. Mesenchymal stem cells (MSCs) from the
bone marrow of mice were used to produce exosomes with low immunogenicity [42]. The plasmid
which expressed lysosome-associated membrane glycoprotein 2b (Lamp2b), an exosomal membrane
protein [32], fused with GFE1 (CGFECVRQCPERC) targeting peptide for pulmonary vascular
endothelial cells [43] on the extra-exosomal N terminus of Lamp2b was constructed. Then, the
corresponding expression vectors were transfected into MSCs to produce Lamp2b-GFE1 fusion protein,
which was exposed on the surface of exosomes. The transfected MSCs were purified and incubated
with IPI549 (a PI3Kγ inhibitor). The GFE-1-positive and IPI549-loaded exosomes (GExoI) were
purified from cell culture supernatants (Fig. 1a). After intravenously injected into a mouse model of
melanoma, GExoI could accumulate at the lung through GFE1 binding to the surface of pulmonary
vascular endothelial cells, and gradually release IPI549 to block the G-MDSCs recruitment and
preventing the PMN formation, leading to suppression of the lung metastasis of melanoma (Fig. 1b).
Collectively, this is the first proof-of-concept attempt to block tumor metastasis based on the tumor
metastasis organotropism and the local PMN inhibition, which will serve as a powerful strategy for
postoperative metastasis of tumor.

**Results and discussion**

1. **Characterization of GExoI**

MSCs were isolated from the bone marrow of mice, which were positive for CD29, CD44, and
CD105, but negative for CD34 and CD45 (Supplementary Fig. 1). The purity of MSCs in culture was
up to 93%. To generate GFE1-positive exosomes, we fused the GFE1 peptide to the extra-exosomal N
terminus of murine Lamp2b protein by introducing the pEGFP-C1-GFE1-Lamp2b plasmid into MSCs.
The level of GFE1-Lamp2b mRNA was assessed at 24 h after transfection using reverse transcription
polymerase chain reaction (PCR). Relative to untransfected MSCs, the transfected MSCs expressed a
high level of GFE1-Lamp2b message RNA (Supplementary Fig. 2). Meanwhile, the transfected MSCs
were further incubated with IPI549 for another 24 h. Then, GFE-1 positive and IPI549-loaded
exosomes (GExoI) were achieved from the culture supernatants through ultracentrifugation. The GFE-1
negative exosomes (aExo), GFE-1 positive exosomes (GExo) and GFE-1 negative but IPI549-loaded
exosomes (aExoI) were similarly prepared as controls. Transmission electron microscopy (TEM) and
cryo-TEM (Fig. 2a) images clearly revealed typical exosome structures of $\alpha$Exo, $\alpha$Exo I, $\beta$Exo I and $\varphi$Exo I. Dynamic light scattering (DLS) analysis showed that $\alpha$Exo, $\alpha$Exo I and $\varphi$Exo I had similar hydrodynamic sizes of $116\pm3.3$, $109\pm4.9$, $110.4\pm5.6$ and $114.3\pm4.1$ nm respectively (Fig. 2a). Western blot analysis revealed that $\alpha$Exo, $\alpha$Exo I and $\varphi$Exo I contained the abundant exosome marker proteins, such as TSG101, CD9 and CD63 (Fig. 2b), suggesting that the successful isolation of exosomes from MSCs. To confirm GFE-1 was located on the surface of exosomes, the binding ability of $\alpha$Exo I and $\varphi$Exo I to His-tagged recombinant DPEP1 that is the receptor for the lung-targeting peptide GFE1 was detected by assessing the levels of His-tag in $\alpha$Exo I and $\varphi$Exo I. Western blot analysis showed that $\varphi$Exo I could strongly bind to DPEP1, but no detectable binding was observed in $\alpha$Exo I group (Fig. 2c), supporting the existence of GFE1 on the surface of $\varphi$Exo I. The loading capacities of IPI549 in the $\varphi$Exo I and $\alpha$Exo I were determined as 17.79 and 15.59%, respectively, and the IPI549 release profile was investigated by high performance liquid chromatography (HPLC). The results implied that both $\alpha$Exo I and $\varphi$Exo I showed similar sustained release profiles, and approximately $83.26\pm1.89$ % of total IPI549 were slowly released from both exosomes within 24 h. It seems the introduction of GFE1 did not affect the drug release capability of exosomes (Fig. 2d).

Figure 2. Characterization of exosomes. a TEM and Cryo-TEM images as well as hydrodynamic sizes of $\alpha$Exo, $\alpha$Exo I, $\beta$Exo I and $\varphi$Exo I. Scale bar, 50 nm. b Western blot analysis of TSG101, CD9 and
CD63 from IPI549-primed MSCs, GExo, BExo, BExoI and GExoI. Western blot analysis of BExoI and GExoI binding with DPEP1 using an anti-6x His-tag. IPI549 release profiles of BExoI and GExoI in the phosphate-buffered saline (PBS) over 24 hours. Data are expressed as means ± SD.

2. Cellular tropism of GExoI

The in vitro biocompatibility of GExoI was assessed by CCK8 assay in murine macrophage cells (RAW 264.7) and human umbilical vein endothelial cells (HUVEC). After 24 h exposure to different concentrations of BExoI and GExoI, the viability of cells was found to be weakly affected (Supplementary Fig. 3 and 4). GFE1 peptide has been reported to efficiently target pulmonary vascular [44]. In order to explore the ability of GExoI targeting to pulmonary vascular, pulmonary vascular endothelial cells (MPVEC) and lung epithelial cells (MLE12) were chosen to be treated with PKH26 (a red fluorescent dye)-labeled BExoI and GExoI. Cellular fluorescence images showed that GExoI could be efficiently taken into MPVEC rather than MLE12, presenting potent red fluorescence, while BExoI had low uptake in both MPVEC and MLE12 (Fig. 3a), which reveals the higher affinity of GExoI compared with BExoI toward the pulmonary blood vessels.

The inhibition effect of GExoI on G-MDSCs migration was investigated in a G-MDSCs/CXCL1 transwell system, where G-MDSCs were cultured in the upper chamber and CXCL1 as chemokine to recruit G-MDSCs was loaded in the lower chamber (Fig. 3b). Various exosomes (GExo, BExo, BExoI and GExoI) were incubated with G-MDSCs for 4 h in the upper chamber. G-MDSCs recruited into the lower chamber were counted by flow cytometry. The results revealed that the numbers of G-MDSCs migration in BExoI and GExoI group were less than those in BExo and GExo group (Fig. 3c), and the percentages of migrated G-MDSCs in BExoI and GExoI groups were only 8.50±2.04 and 7.77±1.76 % (Supplementary Fig. 5), and the PI3Kγ expression of G-MDSCs in the upper chamber was down-regulated after both BExoI and GExoI treatments (Fig. 3d). All these results suggest IPI549-loaded exosomes can significantly inhibit the migration of G-MDSCs. Furthermore, to investigate the targeted transporting ability of GExoI to pulmonary vascular endothelial cells, a G-MDSCs/MPVEC/CXCL1 transwell system was established, while G-MDSCs and BExoI- or GExoI-treated MPVEC were cultured in the upper chamber while CXCL1 was in the lower chamber (Fig. 3e). Because GExoI could target to MPVEC but BExoI could not, we hypothesized only GExoI-treated MPVEC carried IPI549 but BExoI-treated MPVEC did not. Encouragingly, the number of G-MDSCs migration in GExoI-treated
MPVEC group was decreased but the number of αExo\textsuperscript{I}-treated MPVEC group was not (Fig. 3f), and the percentage of migrated G-MDSCs in αExo\textsuperscript{I}-treated MPVEC group decreased to 11.67±1.25% (Supplementary Fig. 6), which is ascribed to the targeted release of IPI549 of αExo\textsuperscript{I} in MPVEC. Moreover, the PI3Kγ expression in G-MDSCs in the upper chamber also was down-regulated in αExo\textsuperscript{I}-treated MPVEC group (Fig. 3g). All above results suggest that αExo\textsuperscript{I} can mediate the targeted delivery of IPI549 to pulmonary vascular endothelial cells and durably inhibit the migration of G-MDSCs by downregulating PI3Kγ.

Figure 3. Cellular tropism of αExo\textsuperscript{I}. a Cellular uptake of αExo\textsuperscript{I} and βExo\textsuperscript{I} as assessed by fluorescence

Figure 3. Cellular tropism of αExo\textsuperscript{I}. a Cellular uptake of αExo\textsuperscript{I} and βExo\textsuperscript{I} as assessed by fluorescence
microscopy. DAPI was used to stain the nuclei (blue). BExo and GExo were stained with PKH26 (red).

Scale bar, 50 µm. b Establishment of G-MDSCs/CXCL1 transwell system. Migration number of G-MDSCs in G-MDSCs/CXCL1 transwell system as assessed by flow cytometry. d Western blot analysis of PI3Kγ expression in G-MDSCs of G-MDSCs/CXCL1 transwell system. e Establishment of G-MDSCs/MPVEC/CXCL1 transwell system. f Migration number of G-MDSCs in G-MDSCs/CXCL1 transwell system as assessed by flow cytometry. g Western blot analysis of PI3Kγ expression in G-MDSCs in G-MDSCs/MPVEC/CXCL1 transwell system. Data are expressed as means ± SD.

3. Enhanced pulmonary targeting ability and bioavailability of GExo

Encouraged by above in vitro results, the in vivo biodistribution of GExo was then taken further investigation. The pKH26-labeled BExo or GExo was injected intravenously to healthy C57BL/6 mice, and the fluorescence images of the major organs showed that BExo and GExo mainly distributed in the liver, kidney and lung, among which GExo had the highest accumulation in the lung while BExo had the highest one in the liver (Fig. 4a and b). This result reveals the excellent pulmonary targeting ability of GExo, which is attributed to GFE1 peptide on the surface of GExo. To further determine the detailed distribution of BExo and GExo in the lung tissue, the lung slices were stained with FITC-CD31, a vascular endothelial cell marker. Fluorescence microscopy image showed that the red fluorescence of pKH26-labeled GExo was bound to the pulmonary vasculature, however, only a few of pKH26-labeled BExo distributed irregularly throughout the lung slice (Fig. 4c). Next, the biodistributions of IPI549 in different organs of mice was quantitatively analyzed by HPLC. Treatments with BExo or GExo could increase the accumulation of IPI549 in the liver (Supplementary Fig. 7), kidney (Supplementary Fig. 8) and lungs (Fig. 4d) of mice as compared with treatment with free IPI549, and more importantly, GExo compared with BExo could more significantly increase the accumulation of IPI549 in the lung. This result demonstrates GExo can enhance the accumulation of IPI549 in the pulmonary vasculature by binding to vascular endothelial cells in the lung. To evaluate the pharmacokinetic profile of IPI549, the time-dependent IPI549 plasma concentration of mice injected with free IPI549 or exosomes (BExo and GExo) was investigated by HPLC. Fig. 4e showed that the maximum IPI549 plasma concentration (C max) of mice with free IPI549 treatment peaked at 20 min post-injection, which was 0.38 µg mL⁻¹, and then decreased rapidly. The C max of mice with BExo or GExo treatment peaked at 2 h post-injection and was improved to 0.72 µg mL⁻¹ (for GExo) or 0.78 µg mL⁻¹ (for BExo). Compared
with free IPI549, both αExo\(^i\) and βExo\(^i\) also contributed to the higher bioavailability and slower metabolism rate.

**Figure 4. Pulmonary targeting of αExo\(^i\).** a Ex vivo fluorescence images of the major organs (heart, liver, spleen, lung, and kidney) from mice at 24 h postinjection with PBS, PKH26-labeled αExo\(^i\) or
Иммунфлюоресцентные изображения лёгких, окрашенных маркером венозных эндотелий CD31 (зелёный). Шкала, 100 μм.

Распределение IPI549 в легких мышей, как описано в Фиг. 4а. е Плазменная концентрация-время кривой IPI549 в плазме мышей после单一 intravenous введения свободного IPI549, aExoI или αExoI (эквивалент концентрации 0.2 мг IPI549/кг мыши).

Данные выражены как среднее ± SD. n = 5 биологически независимых животных в группе.

4. Терапевтическая эффективность αExoI для подавления постоперационного метастазирования меланомы

Первичная ткань опухоли может индуцировать спонтанное развитие PMN, и метастазы будут формироваться даже если опухоль в теле удалена [10]. Для оценки ингибиторной эффективности αExoI против формирования PMN в легких, B16/F10 клеток-дескрипторов ткань экссудированного среды (TCM) модели стимулировался мышей модель был установлен. ТКМ использовался как тканевая субстанция был впервые введена внутривенно в нормальных C57BL/6 мышей, и свободный IPI549, aExoI или αExoI был введен внутривенно в то же время, следуя внутривенной инъекции B16/F10 клеток (Фиг. 5а). PMN характерные гены экспрессии в легких, включая Bv8, S100a8, S100a9, и MMP9, которые были отмечены в реальной времени PCR. Результат показал, что лечение TCM одинично значимо увеличил экспрессию Bv8, S100a8, S100a9, и MMP9 в легких по сравнению с нормальными мышами (Справочные Фиг. 9). Однако, дополнительное лечение с IPI549, aExoI или αExoI уменьшило их экспрессию, где αExoI показало наиболее потенциальную производительность, предполагая αExoI может эффективно ингибировать развитие PMN в легких. Дальнейшие, метастатические узелки в легких были подсчитаны. Только TCM-терапевтические мыши показали наиболее метастатические узелки, ускоряя метастазирование, но дальнейшее лечение с IPI549, aExoI или αExoI вызвало уменьшение метастатических узелков, показывая ингибирование метастазирования (Фиг. 5b и 5c). Особенно, αExoI вызвал уменьшение метастатических узелков, показывая лучшую ингибируя активность против метастазирования. Все вышеупомянутые результаты указывают, что αExoI может значительно ингибировать метастазирование опухоли стимулируя ПМН в легких.
Figure 5. Suppressed metastasis by $\text{g} \text{Exo} ^{\text{i}}$. 

a. Treatment schedule and timeline for TCM stimulation, treatments with various drug formulations, and lung metastasis evaluation. Days were recounted from day 0 after all TCM stimulations and treatments.

b. The metastatic nodules in the lungs of control mice without injection with TCM and mice injected with TCM + PBS, TCM + IPI549, TCM + $\text{b} \text{Exo} ^{\text{i}}$, or TCM + $\text{g} \text{Exo} ^{\text{i}}$.

c. Comparison of the number of metastatic nodules in the lungs of control mice and mice injected with different treatments.

d. Tumor inoculation and tumor removal timeline.

e. Metastatic nodules in control mice and mice injected with different treatments.

f. Comparison of the number of metastatic nodules in control and treated mice.

g. Histological sections showing the metastatic nodules in control and treated mice.
TCM + న్యాయం. All mice were sacrificed at 2 weeks after a tail vein injection with B16F10 cells. The
black point represented visible metastatic nodules in the lung. c Average number of macroscopic lung
metastatic nodules in mice treated as in Figure 5B. d Schematic illustration of postoperative lung
metastasis models and therapies. e Images of the lung tissue from postoperative lung metastasis mice
treated with PBS, IPI549, aExoI or cExoI. f Average number of macroscopic lung metastatic nodules in
mice treated as described in Figure 5E. g H&E-stained tissue sections of lung excised at the end of
treatments. Scale bar, 100 µm. For e-g, mice whose subcutaneous tumors (100 mm³) were completely
excised were intravenously injected with B16/F10 cells at 7 days post-surgery. PBS, free IPI549, aExoI
or cExoI was injected intravenously to the mice every two days after injection with B16/F10 cells. All
mice were sacrificed at 2 weeks after intravenous injection with B16F10 cells. Data are expressed as
means ± SD. n = 5 biologically independent animals per group.

To finally determine whether cExoI can prevent the postoperative tumor metastasis, the postoperative
lung metastasis model was generated in C57BL/6 mice. B16/F10 cells were first subcutaneously
injected in C57BL/6 mice. When the tumor volume reached about 100 mm³, the tumor was completely
excised, followed by intravenous injection of B16/F10 cells at 7 days post-surgery. PBS, free IPI549 or
the exosomes (aExoI and cExoI) were injected intravenously to the mice every two days after injection
of B16/F10 cells (Fig. 5d). Compared to the mice treated with free IPI549, the number of macroscopic
metastatic nodules of both exosomes-treated mice was dramatically decreased, where cExoI still
showed more significant metastases inhibition than aExoI (Fig. 5e and 5f). From the hematoxylin-eosin
(H&E) staining of the lung metastatic lesions (Fig. 5g), metastatic nodules and inflammation could be
distinctly detected in PBS-treated mice. While treatments with free IPI549 or aExoI could decrease the
nodules and inflammation in the lung, there was barely the nodules and inflammation in the lung of
cExoI-treated mice, indicating the remarkable anti-metastatic efficacy of cExoI.

5. Inhibited G-MDSCs accumulation in the lung by cExoI to prevent PMN formation

It has been demonstrated that G-MDSCs play a vital role in promoting metastases in tumor-bearing
mice, and their accumulation in premetastatic organs is a complex process that is regulated mainly by
CXCLs factors (CXCL1, CXCL2, CXCL5, and CXCL8)[23]. Because of the wide range of factors, it
is difficult to achieve anti-tumor metastasis by simply inhibiting the expression of a certain factor [46].
Here, we first design an anti-accumulation strategy to inhibit the chemotactic migration of G-MDSCs into the lung by blocking the CXCLs/CXCR2/PI3Kγ signaling of G-MDSCs. To investigate the influence of αExoI on G-MDSCs recruitment before PMN formation, the percentage of G-MDSCs (CD11b^+Ly6G^+) in the lung of the postoperative lung metastasis mice model was analyzed by flow cytometry. **Fig. 6a and Supplementary Fig. 10** show that IPI549 and αExoI could reduce the percentage of G-MDSCs in premetastatic lungs, but αExoI even decreased the percentage close to the normal level of healthy mice, which is because on the one hand αExoI has a longer circulation time than IPI549, capable of more effectively blocking the chemotactic migration of G-MDSCs, and on the other hand, αExoI has a higher drug delivery efficiency than αExoI, thus releasing the stronger anti-recruitment ability. Moreover, the expression of PI3Kγ in G-MDSCs from premetastatic lungs of mice with different treatments was observed to show the same trend as the percentage of G-MDSCs did (Fig. 6b).

G-MDSCs can suppress the immunity by perturbing both innate and adaptive immune responses, such as promoting the generation of monocytic myeloid-derived suppressor cells (mo-MDSCs), skewing macrophage towards an M2 phenotype, and blocking the activation and proliferation of CD8^+ T cells [47, 48]. Then, the impact of αExoI on the function of G-MDSCs was further assessed. The percentages of the major immune cells in the lung tissues were detected by flow cytometry in the lung of the postoperative lung metastasis mice model. The percentages of mo-MDSCs (CD11b^+Ly6C^+) and M2 macrophages (F4/80^+CD206^+) in the lungs of the αExoI-treated mice decreased most significantly, which probably is because the decreased G-MDSCs could cause the decrease of mo-MDSCs generation and impairment of macrophage polarization towards an M2 phenotype (**Fig. 6c and Supplementary Fig. 11**). The percentage of CD4^+ T cells in the lungs of free IPI549-, αExoI-, and αExoI-treated mice were increased, compared with that of PBS-treated mice (**Fig. 6d and Supplementary Fig. 12**), indicating that the reduction of G-MDSCs can achieve the remodeling of acquired immunity. Moreover, the percentage of CD8^+ T cells in the lungs of the αExoI-treated mice increased most significantly (**Fig. 6d and Supplementary Fig. 13**). Based on the above results, the composition ratio of various immune cells in the premetastatic lung was changed by the reduction of G-MDSCs. IFNγ coordinates multiple protective functions to enhance the immune response to infection and cancer [49] and IL-10 is a pleiotropic anti-inflammatory cytokine that induces immunosuppression and assists in escape from tumor immune surveillance [50]. The lung homogenate of postoperative lung metastasis mice model...
was collected at the end of various treatments for detection of IFNγ and IL10 by enzyme-linked immunosorbent assay (ELISA). As shown in Figure 6e and f, aExoI could induce the highest level of IFNγ among the four treatment groups and the level of IL10 close to that in healthy mice, due to the targeted IPI549 delivery ability of aExoI. Taken all together, the reduction of G-MDSCs by aExoI can create an immune activated microenvironment in the premetastatic lung.

Figure 6. Prevented PMN formation by aExoI in the lung of postoperative lung metastasis mice. a Flow cytometry analysis of the percentage of G-MDSCs. b Western blot analysis of the PI3Kγ expression in G-MDSCs. c Flow cytometry analysis of the percentages of mo-MDSCs and M2 macrophages. d Flow cytometry analysis of the percentage of CD4+ T cells and CD8+ T cells. e, f ELISA analysis of the level of IFNγ e and IL10 f. For Figure 6a-f, mice whose subcutaneous tumors (100 mm³) were completely excised were intravenously injected with B16/F10 cells at 7 days post-surgery. PBS, free IPI549, aExoI and aExoI were injected intravenously to the mice every two days after injection with B16/F10 cells. All mice were sacrificed at 2 weeks after intravenous injection with
B16F10 cells. And the lungs of the mice were used to prepare single-cell suspensions and lung homogenates for flow cytometry and ELISA analysis. Data are expressed as means ± SD. n = 5 biologically independent animals per group.

6. In vivo biocompatibility of GExo

In vivo biocompatibility of GExo was assessed by histological examination of major organs and blood chemistry tests. No noticeable damage was found in H&E stained tissue sections of major organs (heart, liver, spleen, lung and kidney) of mice at the end of treatments with IPI549, aExo or GExo (Fig. 7a). Serum biochemical indices of aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CREA) and blood urea nitrogen (BUN) in the mice treated with IPI549, aExo or GExo were not significantly different from those in the healthy mice (Fig. 7b-e), indicating that this therapeutic strategy may not cause obvious damage to the liver and spleen. Moreover, no body weight loss was found in all the mice groups during the treatment process in the postoperative lung metastasis mice model (Fig. 7f). Taken all together, GExo is a biocompatible choice for the suppression of metastasis.

Figure 7. In vivo biocompatibility of GExo. a H&E staining of major organ sections of mice treated...
with IPI549, \textit{aExo} or \textit{gExo}. Scale bar, 200 µm. \textit{b-e} Levels of AST \textit{b}, ALT \textit{c}, CREA \textit{d} and BUN \textit{e} in serum. \textit{f} Mice body weight curves during the treatment period. Data are expressed as means ± SD. \( n = 5 \) biologically independent animals per group.

Discussion

Functional exosomes (\textit{gExo}) were designed to suppress the lung metastasis of melanoma by preventing the PMN formation. \textit{gExo} that presented GFE1 peptide on the surface of exosomal membrane and loaded IPI549 inside the exosomes showed the significant targeting ability to pulmonary vascular endothelial cells and inhibit G-MDSCs accumulation in the premetastatic lung. In transwell system, \textit{gExo} targeting to MPVEC could release IPI549 to inhibit the migration of G-MDSCs and decrease their PI3K\( \gamma \) expression. In postoperative mice model, intravenously injected \textit{gExo} exhibited a significant aggregation effect in the lung PMN, and transformed immunosuppressive microenvironment into immunostimulative microenvironment by decreasing the percentages of G-MDSCs, mo-MDSCs and M2 macrophage and increasing the percentages of CD4\(^+\) T cells and CD8\(^+\) T cells, leading to the effective inhibition on tumor metastasis. Collectively, we provided herein a proof of concept that early blocking the PMN formation using organ-specific exosomes would afford a potent platform of anti-tumor organ specific metastasis.

Materials and methods

1. Isolation of primary bone marrow MSCs. Primary bone marrow MSCs were isolated from 2 or 3 week old C57BL/6 mice as previously described[39]. Briefly, bone marrow cells from the femur and tibia were flushed thoroughly with a 26-gauge syringe needle inserted into the marrow cavity. Afterwards, the diaphyses of femur and tibia were chopped into small sclerites and digested with collagenase II for 2 h at 37°C in a shaker, 100 rpm min\(^{-1}\). During digestion, bone marrow cells were filtered using a 70 µm strainer filter and centrifuged at 200 g for 5 min. The cell pellet was resuspended in C57BL/6 MSC complete culture medium (Cyagen Biosciences Inc, United States) and seeded in culture dishes. After digestion, bone chips were washed 3 times with basic DMEM/F-12 and seeded
into the culture dishes to co-culture with the bone marrow cells at 37°C containing 5% CO2. After
being seeded for 3 days, MSCs attached to the bottom of culture dishes and the non-adherent cells were
eliminated through the exchange of the cell medium at 72 hours and every 2 days thereafter. Then the
MSCs were identified by stained with anti-CD44-PE (clone IM7, Biolegend), anti-CD45-PE (clone
30-F11, Biolegend), anti-CD105-PE (clone MJ7/18, Biolegend), anti-CD34-PE (clone SA376A4,
Biolegend) and anti-CD29-FITC (clone HMβ1-1, Biolegend) by flow cytometry (BD Biosciences,
AccuriC6). The MSCs from passage 3 to passage 8 were implemented to extract exosomes.

2. Preparation of $\alpha$Exo$^I$ and $\beta$Exo$^I$. The pEGFP-C1-GFE1-Lamp2b plasmid was constructed by the
Beijing Genomics institution (China). $1 \times 10^7$ MSCs were seeded into 100 mm dish. After 24 h, the
MSCs were transfected with 8 μg pEGFP-C1-GFE1-Lamp2b plasmid using Lipofectamine 2000
transfection reagent (Invitrogen, USA). After 24h transfection, the transfected MSCs were washed 3
times with PBS and cultured for another 24 h in media with exosome-free serum containing 40 μg mL$^{-1}$
IPI549. The exosomes were purified using a gradient centrifugation protocol according to the literature
[51]. The cell culture supernatant containing exosomes was harvested by centrifugation at 200 g for 5
min to eliminate cells. The supernatant was then centrifuged at 12,000 g for 45 min to remove dead
cells and cell debris. The resulting supernatant was further centrifuged at 100,000 g for 90 min
(QPTimaMAX-XP Ultra-High, Beckman Coulter, USA). The pellets were washed with large volume of
cold PBS and centrifuged at 120,000 g for 70 min again to ensure maximal exosome purity. All
centrifugation procedures were performed at 4 °C. The pellet of $\alpha$Exo$^I$ was resuspended in PBS and
stored at -80°C. $\beta$Exo$^I$ was prepared using the similar procedure except using the pEGFP-Lamp2b
plasmid instead of the pEGFP-C1-GFE1-Lamp2b plasmid. $\alpha$Exo and $\beta$Exo were prepared using the
similar preparation procedure of $\alpha$Exo$^I$ and $\beta$Exo$^I$, respectively, except without IPI549 incubation.
3. Characterization of exosomes. The primary size and morphology of exosomes were examined using transmission electron microscope (JEM-2010) and cryo-TEM (JEOL JEM-3200FSC). Hydrodynamic size of purified exosomes was determined using Malvern Zetasizer (Nano ZS, Malvern, USA). To confirm successful isolation of exosomes from MSCs, the presence of TSG101, CD63 and CD9 on the exosome surface were measured by western blotting as mentioned by previous description [52]. The primary antibodies were shown below: anti-CD9 (Abcam, UK), anti-CD63 (Abcam, UK), anti-TSG101 (Abcam, UK). The amount of IPI549 loaded into exosomes was measured by a high performance liquid chromatography (HPLC, Waters, USA) at 254 nm. 25 μg μExoI or γExoI in a 1.5 mL centrifuge tube was heated at 75°C to evaporate solvent. Then, 100 μL of acetonitrile was added and the mixture was vortexed, sonicated and then centrifuged at 12,000 rpm for 10 min. The supernatant was collected and filtered through 0.22 μm microfiltration membrane. 20 μL of aliquots was injected into the HPLC system. The chromatographic conditions were as follows: the column was C18 column (4.6 × 250 mm, 5 μm); the mobile phase consisted of acetonitrile: 0.1% formic acid (35:65); the column temperature was 25°C and the detection wavelength was 254 nm [28, 53]. The loading capacity and encapsulation efficiency of IPI549 were calculated using the following formula, where W1 was the weight of the IPI549 enveloped in the γExoI, W was the weight of the exosomes, and W0 was the initial amount of IPI549 added to the culture medium.

\[ LC\% = \frac{W1}{W} \times 100\% \]

\[ EE\% = \frac{W1}{W0} \times 100\% \]

4. Release assessment of IPI549 from exosomes

129.6 μg μExoI or 112.4 μg γExoI (containing 20 μg IPI549) suspended in 1 mL of PBS containing 0.5% carboxymethyl cellulose (CMC) and added to the dialysis bag with a 3500 molecular weight (Mw)
cutoff. Then, the dialysis bag was immersed in a flask containing 200 mL of PBS and 0.5% anionic surfactant sodium dodecyl sulfate (SDS) at 37°C. Samples were taken at time points from inside the flask, and the amount of released IPI549 was analyzed HPLC.

5. Identification of GFE1 expression on exosomes. 10 μg of His-tagged recombinant DPEP1 (Creative BioMart, USA) was incubated with 100 μg of GExoI or BExoI for 4 h at 4°C. Then GExoI or BExoI was purified by ultracentrifugation. The expression of His-tag in GExoI and BExoI were analyzed by western blot. The primary antibody was shown below: anti-6×His-tag (Abcam, UK)

6. Exosome labeling. The fluorescent dye, PKH26 (Umibio, China) was used to label exosomes. Purified exosomes were incubated in 5 mM PKH26 for 15 min at 37°C, and then ultracentrifuged at 120,000 g for 90 min to remove free dye. After being washed twice in PBS, the PKH26-labeled exosomes were resuspended in PBS prior to use.

7. Cells culture. B16/F10 and RAW 264.7 cells were purchased from the American Type Culture Collection (ATCC). MPVEC and MLE12 were separated from the lung of mice. All cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin, and maintained in 5% CO₂ at 37°C.

8. Animals.

Female C57BL/6J mice (4–6-weeks-old) were purchased from the Animal Experimental Center of Jilin University (Changchun, China) and kept under thermo-regulated, humidity-controlled conditions under a 12 h day/night light cycle provided by the experimental and were fed with standard rat chow and water ad libitum. All animal studies were carried out in Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, the operating procedures of the experimental animals were carried out in accordance with protocols approved by the Committee for Animal Research of Changchun Institute.
9. **Preparation of TCM.**

B16/F10 cells were cultured in DMEM with 10% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin at 37 °C in a humidified atmosphere containing 5% CO₂. When the cells formed a consecutive monolayer, the media was replaced with serum-free DMEM, and the cells were incubated for 24 h. Then, the medium was collected and centrifuged. The supernatant was filtered through 0.22 μm syringe filters and stored at -20°C.

10. **Postoperative lung metastasis mouse model.** For *in vivo* metastasis model, B16/F10 cells (1.0 × 10⁶ cells) were subcutaneously injected at the back of C57BL/6 mice to establish the *in situ* melanoma model. When the tumor size reached 100 mm³ (tumor volume = (length × width²)/2), tumors were surgically excised. Seven days later, mice were intravenously injected with B16/F10 cells (2.0 × 10⁵ cells) and the postoperative pulmonary metastasis mouse model was established.

11. **Cytotoxicity assessment of exosomes.** RAW264.7 cells and HUVEC in logarithmic growth phase were digested into single-cell suspensions and seeded in 96-well plates at 1 × 10⁴ cells per well, and then incubated in 5% CO₂ at 37°C overnight. Then, the medium was replaced with 100 μL of fresh medium containing various concentrations of IPI549, aExo¹ or cExo¹. After another 24 h of incubation, 10 μL of CCK-8 reagent (CCK8, Beyotime, Shanghai, China) was added to each well and then cultured for 2 hours. The absorbance was measured at 450 nm by microplate reader (Bio-Rad, Hercules, CA, USA) using wells without cells as blanks.

12. **In vitro Assessment of targeting ability of cExo¹ to MPVEC.** 1.5×10⁵ MPVEC were incubated with 100 μg mL⁻¹ PKH26-labeled aExo¹ or cExo¹ for 2 h. MLE12 cells used as control were similarly treated. The cells were washed with PBS three times and visualized by fluorescence microscopy.
13. G-MDSCs sorted from the blood and lung of tumor-bearing mice.

To isolate G-MDSCs from blood, 2 week tumor-bearing mice were sacrificed by tail vein injection of 4% EDTA. Blood was collected, and the erythrocytes were eliminated with hypotonic lysis buffer. The remaining cells were collected. To isolate G-MDSCs from lung, single cell suspensions of lungs were prepared by cutting the organs into small fragments, digesting them at 37°C for 1 hour with 1 mg mL⁻¹ collagenase I and then filtering them through a 70 μm cell strainer. Erythrocytes were lysed with Red Blood Cell Lysis Buffer (Beyotime, China). The G-MDSCs were sorted with a Myeloid-Derived Suppressor Cell Isolation Kit using an AutoMACS sorter (Miltenyi Biotec, Germany) according to the manufacturer’s instructions. 1 × 10⁸ cells from blood or lungs were centrifuged at 300 g for 10 min at 4°C. The cell pellets were resuspended in 700 μL of PBS (pH 7.2), 0.5% bovine serum albumin, and 2 mM EDTA. Fetal bovine serum (50 μL) was added, mixed well, and incubated for 10 min at 4°C. After incubation, 100 μL of biotin-conjugated anti-Ly6G antibody was added and the cells incubated for a further 15 min at 4°C. Cells were washed by adding 10 mL of buffer and centrifuging at 300 g for 10 min at 4°C. The labeled cells were resuspended in 800 μL of buffer; then, 200 μL of anti-biotin microbeads was added, mixed well, and incubated for 10 min at 4°C. Cells were washed by adding 10 mL of buffer and centrifuging at 300 g for 10 min at 4°C. The cell pellets were then resuspended in 500 μL of buffer. Then, the cell suspension was loaded onto a MACS column, which was placed in the magnetic field of a MACS Separator. The magnetically labelled G-MDSCs cells were retained in the column and washed from the column.

14. The inhibition of GExo on G-MDSCs chemotactic migration in vitro. 5.0×10⁴ G-MDSCs sorted from the blood of tumor-bearing mice were seeded onto transwell inserts with a polyethylene
terephthalate membrane pore size of 5 μm (Corning) in 24-well plates, while CXCL1 (10 ng mL⁻¹ PeproTech Co., Ltd., USA) was added into the lower chamber. Then, 50 μg mL⁻¹ BExo, GExo, BExoI or GExoI were added to the upper chamber for 4 h. Then the number of G-MDSCs migrated into the lower chamber was counted by flow cytometry (BD Biosciences, AccuriC6). To further evaluate the inhibitory effect of GExoI targeting MPVEC on G-MDSC migration, MPVEC were first incubated with 100 μg mL⁻¹ GExoI or BExoI for 2 h, and then the cells were collected and washed with PBS for 3 times to remove unbound exosomes. 1.0×10⁵ G-MDSCs and 1.0×10⁵ these exosome-treated MPVEC were cultured in the upper chamber while CXCL1 (10 ng mL⁻¹) in the lower chamber for 4 h. Then the number of G-MDSCs migrated into the lower chamber was counted by flow cytometry. The percentage of migrated G-MDSCs = the number of migrated G-MDSCs/ the total number of G-MDSCs.

15. Inhibitory effect of exosomes on PI3Kγ expression in G-MDSCs. G-MDSCs in the upper chamber of transwell system were harvested or G-MDSCs in the lung of postoperative lung metastasis mice treated with PBS, IPI549, BExoI or GExoI through intravenously injection were sorted. Then the G-MDSCs were washed with PBS, and mixed with 50 μL cell lysate buffer (Beyotime, China). Samples were centrifuged at 4 °C and 12,000 rpm, 30 min, and the supernatant was collected for Western blot assay. The proteins of the collected supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose blotting membranes (GE Healthcare Life Science, Boston, Massachusetts). Anti-PI3Kγ (1:1000, Santa Cruz Biotechnology, USA) was incubated with the membrane overnight at 4°C. Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibody (1:1000, Beyotime, China) was incubated with the membranes for 1 h at room temperature. The proteins on the membranes were detected using a Tanon imaging system (5200, Shanghai, China).
16. Pharmacokinetics and distribution of exosomes. The tissue distribution and bioavailability of free IPI549, \(_{B}\)Exo\(^I\) and \(_{G}\)Exo\(^I\) in mice were detected as following method [54]. Briefly, healthy C57BL/6 mice were randomly divided into three groups, namely, free IPI549, \(_{B}\)Exo\(^I\) and \(_{G}\)Exo\(^I\), weighed and intravenously injected with a single dose of various formulations at 0.2 mg kg\(^{-1}\) equivalent to the content of IPI549. Blood samples were periodically collected via eye sinus bleeding and immediately centrifuged to obtain plasma for determining IPI549 content at different time point by HPLC. At 24 h after dosing, all the animals were euthanized and their tissues (liver, kidney, lung) were immediately dissected out and homogenized to extract the IPI549 for HPLC analysis. To further monitor the distribution of \(_{B}\)Exo\(^I\) and \(_{G}\)Exo\(^I\) in mice, the C57BL/6 mice were randomly divided into three groups, PBS, PKH26-labeled \(_{B}\)Exo\(^I\) and PKH26-labeled \(_{G}\)Exo\(^I\), weighed and intravenously administered a single dose of various formulations at 0.2 mg/kg equivalent to the content of IPI549. After 24 h, the hearts, livers, spleens, lung and kidneys of the mice were dissected, and a fluorescence imaging system (Davinch-Invivo) was employed to capture the PKH26 fluorescence images of the organs.

17. In vivo targeting ability assessment

PKH26-labeled \(_{B}\)Exo\(^I\) or \(_{G}\)Exo\(^I\) were intravenously injected into healthy C57BL/6 mice (0.2 mg of IPI549-equiv per kg of body weight), and the treated mice were sacrificed after 24 h. Lung tissues were collected and embedded in opti-mum cutting temperature compound (Sakura; Torrance, CA). The frozen sections were fixed with acetone, incubated with 2 \(\mu\)g mL\(^{-1}\) anti-mouse CD31 antibody (Abcam, UK) overnight at 4\(^{\circ}\)C, and then stained with FITC rabbit anti-goat IgG (H\(+\)L) (1:500 dilution, ABclonal) for 1 h at room temperature. The fluorescent signals of section were detected under fluorescence microscopy.

18. Metastasis prevention assay in TCM-stimulating mice model. Tumor conditioned medium (200
μL) was intravenously injected into C57BL/6 mice one time per day for 3 days. After each TCM injection on the same day, the mice were intravenously injected with IPI549, αExoI and δExoI (0.2 mg kg\(^{-1}\) equivalent to the content of IP549). Then, B16/F10 cells (2.0 \(\times\) 10\(^5\) cells) were intravenously injected after 3 days. The lungs were harvested after 14 days of B16/F10 cells intravenous injection and the number of macroscopic metastatic nodules in the lung was recorded.

19. In vivo efficacy of αExoI preventing PMN formation and against pulmonary metastases

Female C57BL/6 mice with established postoperative lung metastasis were randomly divided into 4 groups: PBS, IPI549, αExoI and δExoI (0.2 mg kg\(^{-1}\) equivalent to the content of IP549). Each intravenous injection was administered every other day (total for 6 doses) through the tail vein. The first treatment was 2 days after the establishment of the postoperative lung metastasis model. Two days after the last injection, animals were sacrificed to harvest lungs, and the number of macroscopic metastatic nodules in each lung was recorded to evaluate the therapeutic effects. Furthermore, single cell suspensions of a piece of the lungs were prepared as described above to detect the percentage of G-MDSCs and mo-MDSCs through staining with anti-CD11b-FITC (clone M1/70, BD), anti-CD45-PE/Cy7 (clone 30-F11, BD), anti-Ly6G-APC/Cy7 (clone 1A8, BD) and anti-Ly6C-PE (clone AL-21, BD), respectively, to detect the percentage of CD4\(^+\)T cells and CD8\(^+\)T cells through staining with anti-CD3-FITC (clone 17A2, Biolegend), anti-CD4-PE (clone GK1.5, Biolegend) and anti-CD8-APC (clone 53-6.7, Biolegend), respectively, and to detect the percentage of M2 phenotype macrophages through staining with anti-F4/80-FITC (clone BM8, Biolegend) and anti-CD206-APC (clone C068C2, Biolegend), respectively, by flow cytometry (BD Biosciences, AccuriC6). A piece of lung was homogenized to examine the level of IFNγ and IL10 using Elisa kit (Biolegend, USA) according to manufacturer’s instruction. Afterwards, a piece of the lungs was fixed in 4%
paraformaldehyde, and then sliced into 5 µm-thick sections. Histological examinations of the lung
slices were performed with H&E staining for visualization of metastatic foci.

20. Toxicity Evaluation in Vivo.

The body weight of each mouse was measured every 2 day from tumors excised. At the end of
systematic administration, major organs (heart, liver, spleen, and kidney) were harvested as mentioned
above and served for H&E staining and aspartate transaminase (AST), alanine transaminase (ALT),
creatinine (CREA) and blood urea nitrogen (BUN) levels of serum collecting from different treated
mice were detected using a commercial kit (Nanjing Institute of Biological Engineering, Nanjing,
China) according to the manufacturer’s protocol.

21. Statistical analysis. All data analyses were performed using GraphPad Prism 6.0 (GraphPad
Software, La Jolla, CA, USA). Data are presented as the mean ± SD. Student’s t-test was used to
analyze differences between two groups. One-way ANOVA was used to perform the multi-sample
analysis followed by the Tukey post hoc test. Differences at P < 0.05 were considered statistically
significant.

Author contributions

Conceptualization, methodology, investigation: Xiaoqing Han; investigation, manuscript revision:
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Conflicts of interest
The authors declare no competing financial interest.

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