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Targeting of immunosuppressive myeloid cells from glioblastoma patients by modulation of size and surface charge of lipid nanocapsules --Manuscript Draft--

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Abstract:	<p>Background: Myeloid Derived Suppressor Cells (MDSCs) and Tumor-Associated Macrophages (TAMs) are two of the major players involved in the inhibition of anti-tumor immune response in cancer patients, leading to poor prognosis. Selective targeting of myeloid cells has therefore become an attractive therapeutic strategy to relieve immunosuppression and, in this frame, we previously demonstrated that lipid nanocapsules (LNCs) loaded with lauroyl-modified gemcitabine efficiently target monocytic MDSCs in melanoma patients. In this study, we investigated the impact of the physico-chemical characteristics of LNCs, namely size and surface potential, towards immunosuppressive cell targeting. We exploited myeloid cells isolated from glioblastoma patients, which play a relevant role in the immunosuppression, to demonstrate that tailored nanosystems can target not only tumor cells but also tumor-promoting cells, thus constituting an efficient system that could be used to inhibit their function.</p> <p>Results: The incorporation of different LNC formulations with a size of 100 nm, carrying overall positive, neutral or negative charge, was evaluated on leukocytes and tumor-infiltrating cells freshly isolated from glioblastoma patients. We observed that the maximum LNC uptake was obtained in monocytes with neutral 100 nm LNCs, while positively charged 100 nm LNCs were more effective on macrophages and tumor cells, maintaining at low level the incorporation by T cells. The mechanism of uptake was elucidated, demonstrating that LNCs are incorporated mainly by caveolae-mediated endocytosis.</p> <p>Conclusions: We demonstrated that LNCs can be directed towards immunosuppressive cells by simply modulating their size and charge and provided a novel approach to exploit nanosystems for anticancer treatment in the frame of immunotherapy.</p>	
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1 **Targeting of immunosuppressive myeloid cells from glioblastoma patients by**
2
3 **modulation of size and surface charge of lipid nanocapsules**
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23 **Abstract**

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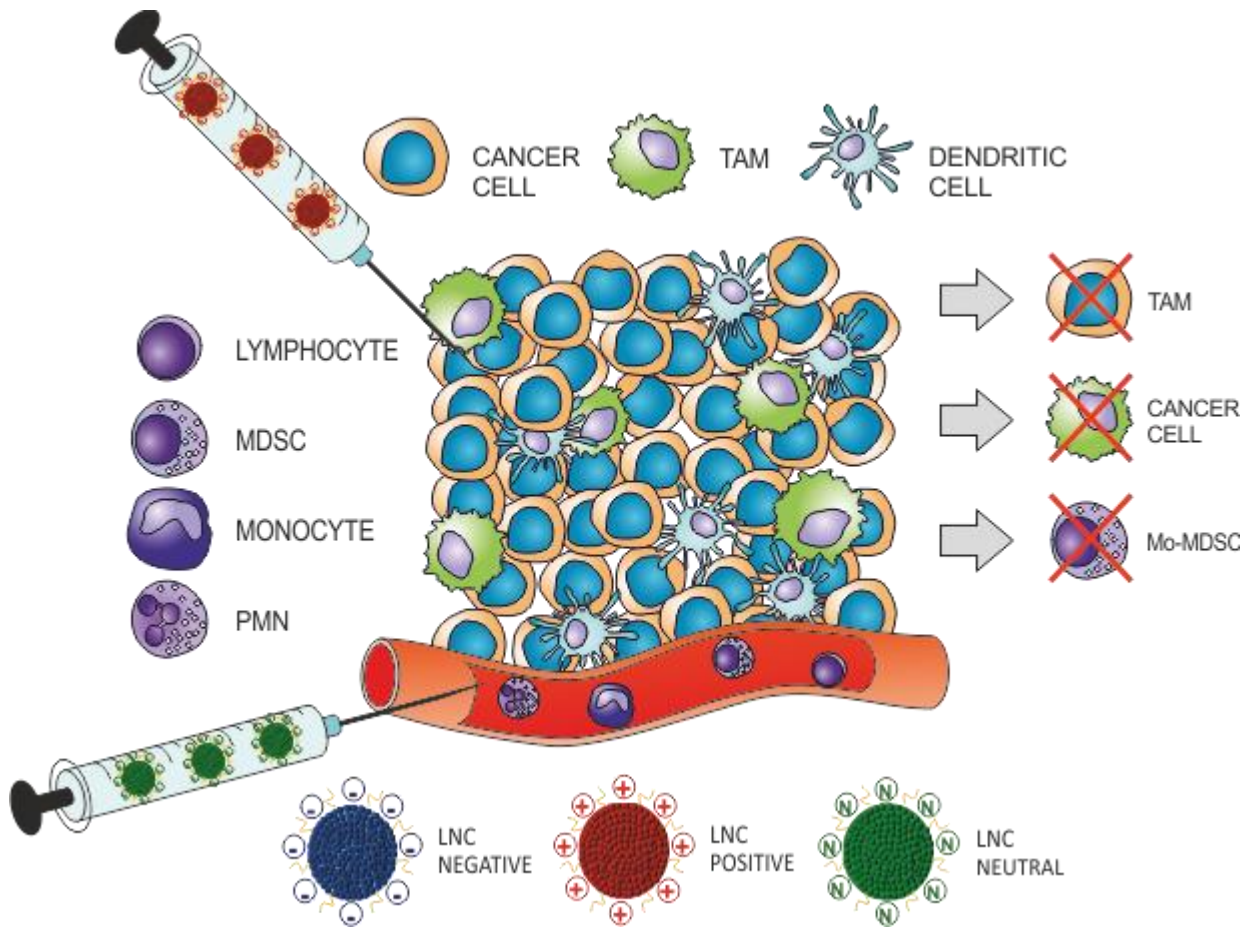
34 **Background:** Myeloid Derived Suppressor Cells (MDSCs) and Tumor-Associated Macrophages
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5 (TAMs) are two of the major players involved in the inhibition of anti-tumor immune response in
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7 cancer patients, leading to poor prognosis. Selective targeting of myeloid cells has therefore become
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9 an attractive therapeutic strategy to relieve immunosuppression and, in this frame, we previously
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11 demonstrated that lipid nanocapsules (LNCs) loaded with lauroyl-modified gemcitabine efficiently
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13 target monocytic MDSCs in melanoma patients. In this study, we investigated the impact of the
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15 physico-chemical characteristics of LNCs, namely size and surface potential, towards
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17 immunosuppressive cell targeting. We exploited myeloid cells isolated from glioblastoma patients,
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19 which play a relevant role in the immunosuppression, to demonstrate that tailored nanosystems can
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21 target not only tumor cells but also tumor-promoting cells, thus constituting an efficient system that
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23 could be used to inhibit their function.
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30 **Results:** The incorporation of different LNC formulations with a size of 100 nm, carrying overall
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32 positive, neutral or negative charge, was evaluated on leukocytes and tumor-infiltrating cells freshly
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34 isolated from glioblastoma patients. We observed that the maximum LNC uptake was obtained in
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36 monocytes with neutral 100 nm LNCs, while positively charged 100 nm LNCs were more effective
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38 on macrophages and tumor cells, maintaining at low level the incorporation by T cells. The
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40 mechanism of uptake was elucidated, demonstrating that LNCs are incorporated mainly by caveolae-
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42 mediated endocytosis.
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47 **Conclusions:** We demonstrated that LNCs can be directed towards immunosuppressive cells by
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49 simply modulating their size and charge and provided a novel approach to exploit nanosystems for
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51 anticancer treatment in the frame of immunotherapy.
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56 **Key words:** myeloid cells, lipid nanocapsules, glioma, myeloid derived suppressor cells,
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58 immunosuppression
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62 **Background**

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263 The recent implementation of nanosystems with different chemical and physical features and loaded
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564 with a variety of compounds offers promising opportunities to target selected cell populations in
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765 cancer [1]. The improved anti-tumor effects of nanomedicine has been widely ascribed to the direct
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1066 cytotoxicity of chemotherapeutics on cancer cells, due to elevated drug concentrations in tumor tissue
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1267 via passive- and/or tumor-targeting and favorable pharmacokinetics. In addition, the manipulation of
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1568 the immune system significantly affects the efficacy of cancer therapies. The contribution of
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1769 nanomedicine to either direct stimulation of the immune system by immunogenic cell death or
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1970 reduction in immunosuppressive populations has the potential to increase antitumor immune response
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2271 by regulating specific pathways within immune cell populations acting on their composition,
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2472 geometry, or surface properties [2-4].

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2773 So far, the effect of size and surface charge has been explored for nanostructures targeting
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2974 tumoral tissues and tumor microenvironment [5]. In regard to the impact of particle size on solid
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3275 tumors, only nanoparticles smaller than 100 nm accumulated efficiently in poorly permeable tumors
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3476 [6], while surface potential influences nanoparticle recognition by blood circulating and tissue
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3677 phagocytes [7]. However, only a few studies have explored the effect of these parameters on
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3978 immunosuppressive myeloid cells.

4179 Myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs)
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4480 constitute two of the main players involved in the induction of immune tolerance in cancer patients.
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4681 MDSCs are a heterogeneous population of myeloid cells able to inhibit innate and adaptive immunity
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4982 in cancer patients and mouse models [8]. Several populations of human MDSCs have been described,
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5183 which, based on their phenotypic and morphological features, can be divided into three main subsets:
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5484 monocytic MDSCs (M-MDSCs), polymorphonuclear MDSCs (PMN-MDSCs) and early-stage
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5685 MDSCs (eMDSCs) [8]. TAMs are particularly abundant in the tumor mass of different tumors and
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5886 can rapidly change their phenotype and function in response to local environmental stimuli, acquiring
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6187 immunosuppressive and pro-tumoral properties [9] and hence they have been associated to poor

88 clinical outcome [10]. Selective targeting of myeloid cells has been advanced as therapeutic strategy
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289 to relieve immunosuppression in patients and increase the response to conventional and
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590 immunotherapy treatments [11]. In this context, identification of a nanosystem selectively targeting
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791 tumor-promoting myeloid cells could represent a new tool to block their activity with the potential to
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92 be used in combination therapy with immune stimulating agents.

1293 We previously demonstrated in a glioma rat model that lipid nanocapsules (LNCs) loaded
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1594 with paclitaxel were able to inhibit multidrug resistance in glioma cells and to reduce tumor
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1795 progression [12, 13]. Moreover, we also showed that an LNC formulation is endowed with a
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1996 preferential targeting of myeloid cells. We found that LNCs loaded with a lauroyl-modified form of
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2297 gemcitabine (GemC12) were able to target M-MDSCs, attenuate tumor-associated
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2498 immunosuppression, and increase the efficacy of adoptive T cell therapy in lymphoma and
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2799 melanoma-bearing mice. Moreover, treatment of monocytes from melanoma patients with GemC12-
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2900 loaded LNCs reduced their immunosuppressive properties *in vitro* [14].
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3101 Starting from these results, in this work we studied the impact of the physico-chemical
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3402 properties, namely size and surface potential, of LNC formulations, already tested in mouse models
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3703 [13-15], to increase their targeting abilities towards MDSCs and TAMs freshly isolated from
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3904 glioblastoma (grade IV glioma, GBM) patients in which several immunosuppressive mechanisms
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4105 have been documented. For example, the expansion of MDSC subsets has been reported in the
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4406 peripheral blood of these patients as compared to healthy donors (HDs) [16], while at the tumor site,
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4607 an abundant infiltrate of myeloid origin has been observed, mainly characterized by macrophages
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4908 [17, 18], which were shown to possess immunosuppressive activity toward T cells [18-21]. TAMs
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5109 comprise both resident microglia (MG) and macrophages of blood origin (bone marrow-derived
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5410 macrophages-BMDM), and we recently demonstrated that in the center of GBM tumor mass BMDMs
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5611 are abundant and endowed with a strong immune suppressive activity [18]. We therefore selected
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5812 GBM as a model to test the targeting of optimized nanosystems towards immunosuppressive cells,
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113 with the future goal of loading these nanoparticles with selected drugs able to deplete or inhibit the
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114 activity of MDSCs and TAMs.

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116 **Results**

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117 **The LNC internalization by blood leukocyte subsets depends on particle size.**

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118 Given the importance of T cell activation and the negative role of myeloid cells in anti-tumor immune
119 response, we rationally modified LNC formulations to target myeloid cells, while reducing the uptake
120 by T cells. LNCs, prepared as previously described, were composed of FDA-approved excipients
121 showing a good safety record and a tunable size (between 20-100 nm) [22, 23]. In this study, we
122 tested LNCs with different physico-chemical properties, i.e. variable size and surface charge, to
123 modulate cell uptake [4, 24, 25].

124

124 First, we tested the uptake of LNCs with 25 nm, 50 nm, and 100 nm size, neutrally charged
125 (Table 1), and loaded with the fluorescent dye DiD (DiD-LNC). Incorporation by leukocyte subsets
126 present in the peripheral blood of HDs was carried out by multicolor flow cytometry, with mAbs
127 directed against markers present on the cell surface of different leukocyte cell subsets and by
128 assessing the signal emitted by DiD in T cells (CD3⁺), monocytes (CD14⁺), B cells (CD19⁺), NK
129 cells (CD56⁺), eosinophils (CD11b⁺/CD16⁻), and polymorphonuclear cells (PMN, CD11b⁺/CD16⁺).
130 Blank-LNCs were used as control. As shown in Figure 1A, the incorporation of 25 nm LNCs was
131 very low in all the considered leukocyte populations, while 50 nm LNCs showed the highest uptake
132 in the analyzed subsets and in particular in monocytes (30.1±3.2%). The 100 nm LNCs did not reach
133 the same uptake of 50 nm LNCs on monocytes (19.4±0.4%) but allowed the reduction in
134 internalization by T lymphocytes (10.5±3.1% with 50 nm LNCs vs 3.3±0.9% with 100 nm LNCs).
135 We excluded from the analysis the 25 nm LNCs and further investigated the internalization properties
136 of neutral 50 nm and 100 nm LNCs, focusing on monocytes and T cells, and increasing the incubation
137 time from 90 minutes to 3 hours in order to reach the highest LNC internalization (Figure 1B). Under

138 these experimental conditions, both LNC formulations reached comparable high levels of
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 139 internalization in monocytes, but the incorporation by T cells was significantly lower when 100 nm
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 140 LNCs were used (Fig. 1B). By calculating the ratio between the signal of DiD in monocytes and T
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 141 cells, we observed that 100 nm LNCs allowed increasing specificity of LNC targeting towards
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 142 monocytes (mean ratio of 4.9 ± 2.7 for 50 nm LNCs vs 11.2 ± 3.8 for 100 nm LNCs) (Figure 1C).
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 143 Therefore, neutral 100 nm LNC formulation was chosen for further experiments.
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145 **Table 1: Size (nm), Polydispersion Index (PI) and Zeta Potential (mV), of LNCs (n>3)**

Formulation	Size (nm)	PI	Zeta Potential (mV)
25 nm LNCs Neutral	25 ± 1	< 0.1	-0 ± 1
50 nm LNCs Neutral	53 ± 4	< 0.1	-4 ± 1
100 nm LNCs Neutral	101 ± 3	< 0.1	-3 ± 0.5
100 nm LNCs Negative	102 ± 3	< 0.1	-20 ± 3
	99 ± 3	< 0.1	$+6 \pm 2$
	102 ± 1	< 0.1	$+16 \pm 3$
100 nm LNCs Positive	92 ± 7	< 0.1	$+25 \pm 2$
	95 ± 8	< 0.1	$+31 \pm 3$

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 148 **Effect of 100 nm LNC surface charge on the internalization ability of peripheral blood**
 149 **leukocytes (PBLs)**
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150 We next set out to assess the surface charge of 100 nm LNCs to increase the specific uptake by
 151 monocytes compared to all the other main leukocyte populations. To this aim, we compared 100 nm
 152 neutral LNCs (-3 mV) with a slightly positive surface charge. The loading of cationic surfactant
 153 DDAB in nanosystems did not alter the size of the systems, while it affected the surface properties of
 154 the LNCs. The physico-chemical characteristics are summarized in Table 2.

155 After 3h of incubation of PBLs with DiD-loaded LNCs (Figure 2A), the internalization by T
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156 cells was very low and comparable in all the tested LNC formulations, while in monocytes the
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157 incorporation was always significantly higher than that of T cells, and had a trend toward an increase
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158 as the positive charge augmented (98.6±1.2% of DiD⁺ monocytes using +31 mV LNCs vs 90.7±4.7%
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159 with neutral LNCs; 82.0±16.5% with +6 mV LNCs; 85.1±12.9% with +16 mV LNCs; 95.6±4.3%
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160 with +25 mV LNCs).
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161 We therefore tested the internalization of 100 nm positive LNCs (+31 mV) by all leukocyte
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162 subsets present in the peripheral blood of HDs and compared the results to neutral LNC formulation
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163 (Figure 2B). Monocytes showed the highest incorporation of positive (+31 mV) LNCs (92.0±1.4%),
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164 but a high uptake was also noticed for B lymphocytes (43.7±19.2%), an effect that was not observed
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165 using neutral LNCs (2.1±0.3%). This could lead to a deleterious consequence on the patient's immune
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166 response if LNCs loaded with a cytotoxic drug were used. We thus selected the 100 nm neutral
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167 surface charge formulation to target monocytes in the peripheral blood and avoid incorporation by B
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168 cells.
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180 **Table 2 – LNCs 25, 50 & 100 nm formulations**

Excipient (mg)	LNC size (nm)		
	25nm	50nm	100nm
Labrafac®	600	1116,8	1800
Kolliphor® HS15	1800	916,8	950
Span 80	300	450	300
MilliQ water	1300	1516,8	950
NaCl	54	54	54
Quenching water	2000	2000	2000

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183 **Targeting circulating immunosuppressive cells in GBM patients by 100 nm neutral LNCs**

184 Data from literature [16] and our own results (Pinton *et al.*, unpublished) indicate that GBM patients
 185 have a significant expansion in circulating MDSC populations. We thus tested the uptake of the 100
 186 nm neutral LNC formulation on leukocyte subsets present in the peripheral blood of these patients,
 187 extending the analysis to three MDSC subsets: two monocytic subsets (identified as CD14⁺/IL4Rα⁺
 188 and CD14⁺/HLA-DR^{low} cells) and one PMN type (CD15⁺/IL4Rα⁺) [8]. Following PBL incubation
 189 with DiD-loaded LNCs, maximum LNC internalization was observed by total monocytes (CD14⁺
 190 cells: 83.0±6.4%) and by monocyte subsets CD14⁺/HLA-DR^{low} (86.4±8,2%) and CD14⁺/IL4Rα⁺
 191 (84.7±6,9%) cells, corresponding to monocytic MDSCs (Fig. 3A), thus highlighting that this
 192 nanocarrier system could efficiently target immunosuppressive myeloid cells in GBM patients, while
 193 sparing lymphocyte subsets that, instead, showed very low uptake. A lower level of incorporation
 194 was observed in PMN-MDSCs, defined as CD15⁺/IL4Rα⁺ cells, as compared to the two monocytic
 195 subsets; it should be noted that this immunosuppressive granulocytic population shows a significantly

196 higher LNC uptake as compared to total PMNs (defined as CD15⁺ cells) (7.6±7% DiD⁺ cells in
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197 CD15⁺/IL4Rα⁺ vs 2.4±1.2% DiD⁺ cells in PMNs), thus reinforcing the efficacy of LNCs in targeting
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198 immunosuppressive cells. To confirm intracellular localization of LNCs, we performed confocal
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199 analysis in isolated peripheral blood mononuclear cells (PBMCs) and PMN fractions, and observed
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200 that DiD-loaded lipid nanoparticles are internalized by monocytes showing cytoplasmic localization,
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201 while no uptake by granulocytes was observed (Fig. 3B).
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203 **Mechanism of LNC internalization by circulating monocytes**

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204 To gain evidence about the mechanisms involved in 100 nm neutral LNC internalization by
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205 monocytes, PBLs from GBM patients were treated with inhibitors of different uptake mechanisms
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206 and their effect was verified on particle internalization. Colchicine was used to inhibit pinocytosis
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207 [26], cytochalasin B as inhibitor of phagocytosis [26], LY294002 and Wortmannin as inhibitors of
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208 fluid phase pinocytosis and FcR-mediated phagocytosis [27, 28], and nystatin as inhibitor of
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209 caveolae-mediated endocytosis [29]. Results indicate that nystatin is the most effective inhibitor,
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210 causing a significant reduction in the uptake of monocytes (Figure 4A). Another inhibitor that showed
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211 a trend toward a reduced LNC internalization by monocytes is colchicine, although its effect did not
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212 reach the same level observed with nystatin, lacking statistical significance. Altogether, these results
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213 indicate that 100 nm neutral LNCs are mainly internalized by monocytes from GBM patients through
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214 caveolae-mediated endocytosis. We also visualized the effect of nystatin on LNC uptake by PBMCs
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215 from three glioma patients, by means of confocal microscopy. Monocytes were enriched by adhesion
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216 onto microscope slides and further selected by nuclear morphology; following nystatin treatment, we
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217 observed a remarkable reduction in the internalization of DiD-loaded LNCs (Fig. 4B), thus
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218 confirming the results obtained by flow cytometry.
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220 **Evaluation of LNC uptake by the cells present in GBM microenvironment**

221 Since 100 nm neutral LNCs efficiently target myeloid cells in the blood of GBM patients, we
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222 evaluated whether the same nanosystem could be incorporated by different cell subsets present in the
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223 tumor microenvironment. In fact, our recent results highlight the presence of an abundant leukocyte
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224 infiltrate of myeloid origin, mainly constituted by macrophages, as a main characteristic in GBM. We
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225 demonstrated that only macrophages of bone marrow origin, and not the resident microglial cells
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226 (MG), were endowed with a strong immunosuppressive activity [18]. In fact, during tumor growth,
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227 macrophages originating from bone-marrow (BMDM) are recruited to the tumor, and can be
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228 distinguished by tissue resident MG through a marker combination in multicolor flow cytometry (Fig.
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229 5A upper panels) [17, 18]. We therefore tested the incorporation of different LNC formulations with
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230 a size of 100 nm, carrying overall positive, neutral or negative charge, in the cell suspension freshly
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2231 obtained from tumor tissue and analyzed the incorporation by BMDM, MG, tumor cells, PMNs, and
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2232 lymphocytes. The maximum LNC uptake was obtained for macrophages (BMDM and MG) and
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2233 tumor cells when positively charged LNCs were used; the low level the incorporation by T cells was
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234 comparable across different formulations. Moreover, both BMDM and MG cells also reached an
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235 uptake significantly higher than PMNs and this LNC formulation also allowed an increased uptake
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236 by tumor cells, evaluated as CD45⁺ cells (Fig. 5A and B).
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Positively charged LNCs show a very high uptake by B cells from the peripheral blood (Fig.2B), but B lymphocytes are not present in GBM tumors (data not shown); therefore, these data indicate that 100 nm positively charged LNCs could be used as a drug-loaded nanosystem to target the main immune suppressive cell subset in these tumors. Moreover, tumor cells also show a significant uptake, thus reinforcing the possibility of using this nanosystem to target both tumor cells and tumor-promoting cells.

66 **Mechanism of LNC internalization by the GBM microenvironment**

245 Given the high ability of tumor macrophages to internalize positively charged LNCs, we analyzed
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246 the mechanism by which these nanoparticles were incorporated by the cells of the tumor
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247 microenvironment. We thus treated the cell suspension obtained after enzymatic digestion of GBM
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248 tumor tissue with uptake inhibitors and added DiD-loaded LNCs to cell suspensions for overnight
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249 incubation. As shown in Figure 6, internalization by both types of macrophages (BMDM and MG)
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1250 was significantly reduced by the addition of nystatin, in line with the internalization observed by
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1251 blood monocytes. A reduction in LNC uptake was also observed using colchicine in both BMDM
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1252 and MG and cytochalasin B in BMDM, but without statistical significance.
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22 **Discussion**

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255 Functionalization of nanosystems offers the opportunity to maximize the protection of associated
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256 drug and the targeting properties of the carrier towards the desired cell populations. In this study, we
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257 optimized LNCs to target immunosuppressive populations in GBM patients, with the aim of
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3258 disclosing innovative applications in the field of immunotherapy. Indeed, as recently shown in two
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359 pioneer studies of personalized vaccination in GBM patients, the immune system can be activated
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360 toward tumor antigens expressed by the tumor but the induction of an immune response does not
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4061 directly translate into a clinical benefit, likely because other critical aspects of the complex interaction
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4252 between tumor and immune systems are not yet defined [30, 31]. From several studies, it appears that
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4263 one of the main obstacles toward a successful anti-tumor immune response is the suppression exerted
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4264 by myeloid cells on cells of the adaptive immune system. Therefore, we tested known LNC
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5065 formulations, with slight modifications in size and charge, capable of targeting immune suppressive
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5266 subsets in cancer patients, but we also evaluated the interactions of LNCs with many subsets of the
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5367 immune system, including those potentially able to mediate cancer regression, like the T
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5268 lymphocytes. To the best of our knowledge, this is the first study to provide a broad picture on the
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5269 impact of different LNC formulations on human immune cell subsets freshly isolated from GBM
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270 patients. We thus extend our previous results focused on immune suppressive myeloid cells in the
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271 blood of melanoma patients to circulating and tumor-associated myeloid cells in GBM patients. The
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272 LNC formulations used in the present work were already tested in mouse models and proved to be
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273 non-toxic, using different administration routes, and effective in reaching tumor site and reducing
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274 tumor growth [13-15].
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1275 Until now, only a few studies addressed LNC targeting towards MDSCs, as a way to either
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1576 deplete them or to induce their maturation. Aptamer (T1) conjugated to liposomal doxorubicin [32]
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1777 or lipid-coated biodegradable hollow mesoporous silica nanoparticle co-encapsulated with all-trans
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1978 retinoic acid (ATRA), doxorubicin and IL-2 [33] showed to have high affinity for both tumor cells
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2279 and PMN-MDSCs [32] or to induce a reduction in the number of MDSCs in the tumor
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2480 microenvironment of mouse models [33]. However, these systems showed no affinity for M-MDSCs
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2781 or macrophages therefore reducing their applicability only to tumors in which PMN MDSCs are
28
2982 mainly involved [32]. Besides, they affected other cell subsets present in the tumor
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3283 microenvironment, and thus cannot be considered a nanosystem with a specific targeting towards
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3484 MDSCs.
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3785 Moreover, Zinc-doped iron oxide nanoparticles modified with polyethylenimine molecules
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3986 and dimercaptosuccinic acid in combination with radiotherapy prolonged survival of CT-2A mouse
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4187 glioma model and were mainly incorporated by TAM/MDSCs, although the definition of these cell
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4488 populations was based only on CD45 and CD11b markers without further characterization [34]. The
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4689 activity of MDSCs was shown to be modulated also using polyarginine nanocapsules carrying the
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4990 chemokine CCL2 and an RNAi sequence targeting C/EBP β , a transcriptional factor fundamental for
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5191 MDSC differentiation and functions [35], thus showing that MDSCs represent an interesting target
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5492 in a nanomedicine approach.
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5693 The idea of using tailored LNCs, encapsulated with selected drugs, to reduce blood monocytes
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5894 stems from the findings that monocytes are actively recruited at tumor site and sustain the
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6195 accumulation of immunosuppressive macrophages in the tumor microenvironment of GBM patients
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296 [18]. Given the high rate of relapse in GBM patients, targeting blood monocytes should be evaluated
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297 as an adjuvant therapy in such patients after surgical resection, to deplete them or to block their
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298 function. This strategy would allow the inhibition of the loop through which GBM tumor attracts
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299 immunosuppressive cells at tumor site and suppresses the anti-tumor immune response. The stability
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300 of neutral 60 nm LNCs loaded with 5-FU in human plasma was already tested and proved that the
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301 system is stable and ~~that~~ protected the drug from rapid degradation, thus confirming the potential use
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302 of LNCs as a tool to target immunosuppressive cells in peripheral blood of patients [36].
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303 The efficacy of LNCs in the treatment of glioma was demonstrated in GL261 glioma-
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304 bearing mice following stereotactic injection. Multifunctional lipid nanocapsules designed to
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21
305 combine the activity of the cytotoxic drug paclitaxel (PTX) with the immunostimulant CpG were
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306 intratumorally administered and were able to increase the survival of mice compared to control, i.e.
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307 the free Taxol®, or PTX-loaded LNCs. This effect was also confirmed by magnetic resonance
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308 imaging, which revealed the reduction of tumor growth in the treated animals [13]. Moreover,
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309 Vanpouille-Box *et al.* demonstrated that lipid nanocapsules loaded with rhenium-188 (LNC¹⁸⁸ Re-
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310 SSS) implanted in the brain of a rat orthotopic glioma model triggered remarkable survival responses.
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311 A strong activation of myeloid cells assessed by immunohistochemistry was observed in this model
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312 together with the recruitment of natural killer and dendritic cells, thus suggesting an improved
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313 capacity to develop an antitumor immune response [37]. Another study, performed in a murine glioma
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314 model with a different type of nanosystem, *i.e.* cyclodextrin-based nanoparticle (CDP-NP),
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315 demonstrated a predominant uptake of CDP-NP by macrophages and microglia within and around
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316 the tumor site [38].
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317 In the present study we optimized a LNC formulation to target immunosuppressive
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318 BMDMs in the GBM microenvironment, but documented that also tumor cells incorporate a lower
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319 but significant amount of such LNCs, thus demonstrating that this nanosystem might target both
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320 tumor cells and tumor-promoting cells. These results open the road to new strategies of therapeutic
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321 interventions. For example, LNCs could be loaded with a drug inducing immunogenic cell death,
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322 offering the possibility of eliminating both suppressive macrophages and tumor cells, thus reversing
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323 a tolerogenic microenvironment, and providing the rationale for a new treatment of GBM. However,
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324 systemic administration would lead to a high uptake by liver, kidney, and spleen, and would not
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325 guarantee a sufficient uptake by the tumor [39, 40]. Thus, to avoid this problem, an intra-thecal
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326 administration, following tumor resection, could be exploited, in order to maximize local efficacy
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327 and reduce systemic side effects.
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329 **Conclusions**

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330 Immune suppression induced by myeloid cells is one of the main obstacles to an efficient
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331 immunotherapy of cancer and targeting myeloid suppressive cells might lay the ground for improving
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332 the its efficacy in cancer patients. Our study shows that modulation of size and charge of nanosystems
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333 impacts the uptake by human blood circulating leukocytes and tumor-infiltrating cells. We exploited
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334 such properties to optimize targeting of LNCs with the aim to relieve immunosuppression dependent
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335 on myeloid cells present in the blood and tumor microenvironment of GBM patients. This study
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336 represents a proof of principle that different physico-chemical characteristics of a nanocarrier systems
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337 can be exploited in order to target a specific cell subset, while sparing others of therapeutic
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338 importance. This approach could be extended to other cancers and set the ground as a new tailored
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339 anticancer treatment in the frame of immunotherapy.
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341 **Methods**

342 **Patient characteristics**

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343 Patients were recruited at the Department of Neurosurgery, Padova University Hospital, Italy. The
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344 ethical committee of the IOV-IRCCS and of Padova University Hospital approved all experiments
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345 and all patients gave their informed consent. We analyzed peripheral blood from 12 GBM patients
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346 and tumor tissue from 6 GBM patients. The studies were conducted in accordance with the
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347 Declaration of Helsinki. To optimize experimental conditions and to test the internalization properties
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348 of different LNC formulations, peripheral blood of 15 HDs was analyzed.
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350 **Isolation of PBLs and PBMCs from peripheral blood of HDs and GBM patients**

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351 Peripheral blood was collected from HDs and GBM patients and subjected to lysis to remove red
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352 blood cells and viable PBLs were counted. PBMCs and PMNs were isolated as previously described
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353 [41]. Additional information are described in Supplementary Methods.
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21 **GBM tissue processing to obtain a single-cell suspension**

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24 GBM tumors were processed immediately after resection, as described in Supplementary Methods,
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26 to obtain a single cell suspension without erythrocytes and debris.
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32 **Preparation of lipid nanocapsules**

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34 LNC formulation was based on a phase inversion process already described [14, 22]. Different LNCs
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36 having a size of 25, 50, and 100 nm were prepared by varying component amounts according to table
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44 **Physico-chemical characterization of LNCs**

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46 Size, estimated by the average hydrodynamic diameter, polydispersity index (PDI) and zeta (ζ)-
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48 potentials were determined by dynamic light scattering (DLS) using NanoZS® (Malvern
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50 Instruments, Worcestershire, United Kingdom). Electrophoretic mobility was converted to ζ -
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52 potentials using Smoluchowski's equation. Measurements were performed at a 173° angle after
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54 dispersion of 50 μ L LNCs in 2.95 mL MilliQ water to ensure convenient scattered intensity on the
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56 detector. All measurements were performed in triplicate at 25°C with comparable conductivity for ζ -
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58 potential determination.
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LNC incorporation by cell populations in peripheral blood and tumor tissue

PBLs, PBMCs, or tumor cell suspension obtained as previously described were incubated with LNCs at the final DiD concentration of 50 ng/ml for 90 minutes, 3 hours, or overnight at 37°C. As control, Blank-LNC formulations were used. 10 µg/ml Cytochalasin B (Sigma-Aldrich), 100 µg/ml Colchicine (Sigma-Aldrich), 50 µM LY294002 hydrochloride (Sigma-Aldrich), 100 nM Wortmannin (Sigma-Aldrich), and 100 U/ml Nystatin were used to test the mechanism of internalization. At the end of the incubation, cells were stained for flow cytometry analysis. Further details are reported in Supplementary Methods.

Multiparametric flow cytometry

Leukocytes were stained with antibody cocktails in order to define different cell populations as specified in Supplementary Materials and Methods. Data acquisition was performed using LSRII flow cytometer (BD Biosciences) and results were analyzed by FlowJo software (Three Star Inc). Further details in Supplementary Methods.

Confocal microscopy

PBMCs and PMNs were incubated with LNCs and nystatin inhibitor and then prepared for confocal analysis as specified in Supplementary Methods. Samples were analyzed under a laser scanning confocal microscope (Leica TCS SP5, Wetzlar, Germany) equipped with 4 lasers (405nm/ Argon-458,476,488,494,514nm-/ 561nm/ 633nm), and results were analyzed by Las X (Leica MICROSYSTEMS).

Statistical analysis

396 The Mann-Whitney and the Student t-test were used as appropriate and performed by Sigmaplot
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397 software (Systat Software Inc., CA, USA).

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399 **Supplementary information**

400 **Supplementary information** about methods section is available in Additional file 1.

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402 **Abbreviations**

403 ATRA: all-trans retinoic acid; BM: bone-marrow; BMDM: bone marrow-derived macrophages;
404 eMDSC: early-stage MDSC; GBM: glioblastoma multiforme; GemC12: lauroyl-modified form of
405 gemcitabine; HD: healthy donor; LNC: lipid nanocapsule; MG: microglia; MDSC: Myeloid Derived
406 Suppressor Cell; M-MDSC: monocytic MDSC; PTX: paclitaxel; PBMC: peripheral blood
407 mononuclear cell; PMN: polymorphonuclear cell; PMN-MDSC: polymorphonuclear MDSC; TAM:
408 Tumor-Associated Macrophage.

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410 **Declarations**

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414 **Ethics approval and consent to participate**

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417 The ethical committee of the IOV-IRCCS and of Padova University Hospital approved all
418 experiments and all patients gave their informed consent

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420 **Consent for publication**

Not applicable

421 **Availability of data and materials**

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422 Not applicable
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423 **Competing interests**

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8
424 The authors declare that they have no competing interests.
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12
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431 Foundation (Project call, 2017).
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432 **Authors' contributions**
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433 LP, SM, EM, MV, IS, VI performed the experiments and analyzed the data. SM and LP wrote the
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434 manuscript. KM and JPB produced lipid nanocapsules and discussed the data. GL produced lipid
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435 nanocapsules, discussed the data and was involved in manuscript writing. VB and IM discussed data,
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436 made critical revision and participated in manuscript writing. ADP performed neurosurgery, provided
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43
437 clinical information and discussed the results. SM designed the research, handled funding and
45
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438 supervised the work. All the Authors read and approved the manuscript.
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540 **Figure legends:**

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541 **Fig.1** Incorporation of LNCs of different size by PBLs. **a** PBLs from 3 HDs were treated for 90' with
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542 neutral DiD-LNCs of different size (25 nm in black, 50 nm in orange and 100 nm in blue), with DiD
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543 at 50 ng/ml and then stained with mAbs (anti-CD3, anti-CD14, anti-CD19, anti-CD56, anti-CD11b,
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544 anti-CD16) for flow cytometry analysis. Blank-LNCs were used as negative control. **b** PBLs from 3
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545 HDs were treated for 3h with 50 nm and 100 nm DiD-LNCs at a DiD concentration of 50 ng/ml and
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546 stained with anti-CD14 and anti-CD3 mAbs to identify monocyte (black) and T lymphocyte (grey)
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547 uptake. **c** The histogram in panel B shows the ratio between the percentage of DiD⁺ cells among
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548 CD14⁺ and CD3⁺ populations. Mean and standard error (SE) of 3 independent experiments are
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549 reported. Student t-test was performed, * P≤0.05; **P≤0.01; ***P≤0.001.
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550 **Fig.2** Incorporation of differently charged LNCs by leukocyte populations. **a** Mean and SE of three
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551 independent experiments on PBLs obtained from peripheral blood of HDs. 100 nm DiD-LNCs with
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552 different surface charge (from neutral to positive) at 50 ng/ml DiD concentration were incubated for
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553 3h with PBLs; DiD-LNC uptake was assessed on CD14⁺ (in black) and CD3⁺ cells (in blue). Blank-
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554 LNC samples were used as negative control. **b** 100 nm positively charged LNCs (+31 mV, grey) and
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555 neutral LNCs (-3 mV, pink) were incubated with PBLs for 3h at 50 ng/ml DiD concentration. DiD⁺
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556 cells by flow cytometry analysis. Mean and SE of 3 independent experiments are reported. Student t-
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557 test was performed, * P≤0.05; **P≤0.01; ***P≤0.001.
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558 **Fig.3** Uptake of neutral LNCs by leukocyte subsets in the peripheral blood of GBM patients. **a** Mean
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559 and SE of PBLs from 7 GBM patients incubated with 100 nm neutral LNCs for 3h at a DiD
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560 concentration of 50 ng/ml. DiD-LNC uptake was evaluated by flow cytometry. Mann-Whitney test
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561 was performed, * P≤0.05; **P≤0.01; ***P≤0.001. (B) PBMCs and PMNs isolated from 3 GBM
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562 patients were incubated with 100 nm neutral LNCs for 3h at a DiD concentration of 50 ng/ml. Then
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563 cells were washed and plated, nuclei were counterstained with DAPI, and the slides were analyzed
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564 by confocal microscopy. Representative fluorescence images of PBMCs and PMNs are shown at a
1 magnification of 150X **b** Cell size is reported by scale bar (10 μ m).

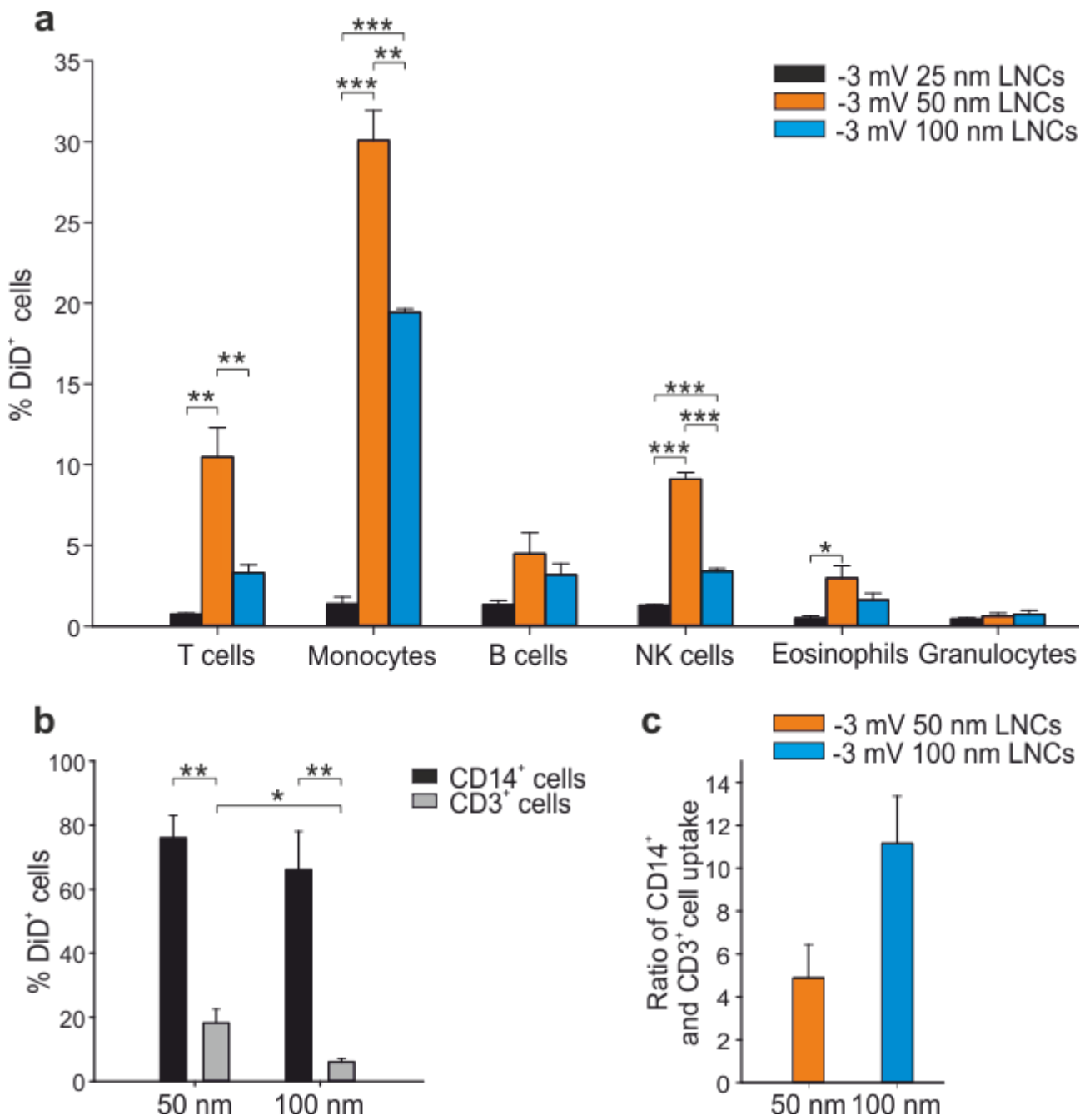
566 **Fig.4** Effect of different inhibitors on LNC uptake by circulating monocytes. **a** PBLs from 3 GBM
2 patients were incubated for 2 hours with 100 μ g/ml colchicine or 10 μ g/ml cytochalasin B, 45 minutes
3 with 50 μ M LY294002, 30 minutes with 100 nM Wortmannin, 15 minutes with 100 U/ml Nystatin.
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574 **Fig.5** Uptake of differently charged LNCs by the cells in GBM microenvironment. **a** Representative
example of flow cytometry analysis on cell suspension from GBM tissue incubated overnight with
100 nm negative, neutral and positive DiD-LNC formulations at 50 ng/ml DiD concentration . The
gating strategy is reported in the upper panels. DiD⁺ cells (blue histograms) were assessed among
BMDM, MG, CD45⁻ cells and compared to the signal of Blank-LNCs (red histogram) among the
same populations. **b** The histograms show mean \pm SE of 3 independent experiments, performed as
described in (A). The percentage of DiD⁺ cells was calculated in BMDM (green), MG (blue), CD45⁻
cells (pink), PMN (yellow), and lymphocytes (grey), setting the gate on Blank-LNC control. PMNs
were gated as CD14⁻/SSC^{high} cells and lymphocytes as CD14⁻/SSC^{low} cells. Student t-test was
performed, * P \leq 0.05; **P \leq 0.01; ***P \leq 0.001.

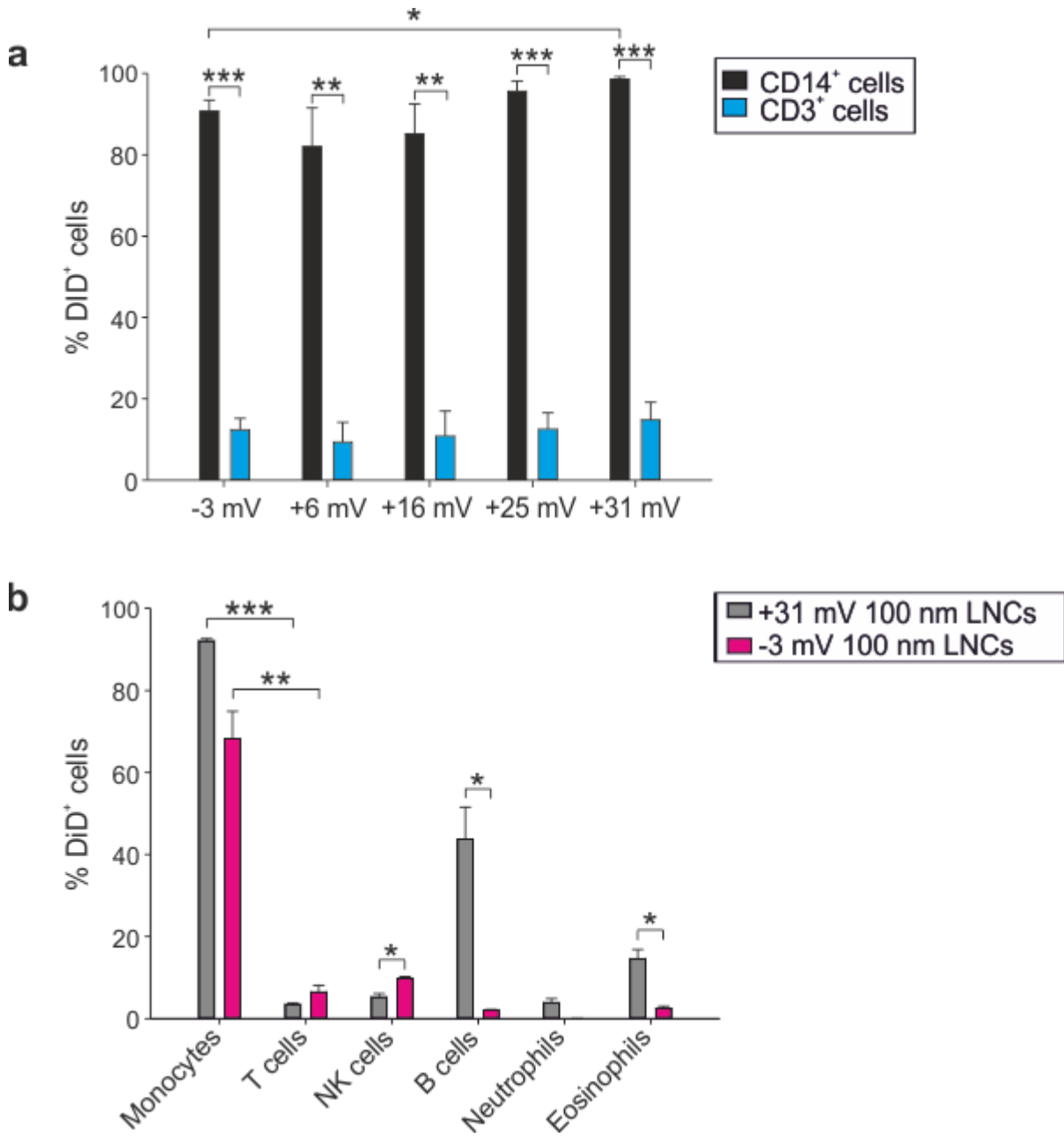
584 **Fig.6** Mechanism of LNC internalization by BMDM and MG. Box plots show the range of DiD⁺ cells
among BMDM **a** and MG **b** in 3 independent experiments. Cells from GBM tissue were incubated
with 100 μ g/ml colchicine, 10 μ g/ml cytochalasin B, 50 μ M LY294002, 100 nM Wortmannin, 100
U/ml Nystatin, DiD-loaded LNCs were added at 50 ng/ml DiD over-night and LNC internalization
was analyzed by flow cytometry.. Student t-test was performed, * P \leq 0.05; **P \leq 0.01; ***P \leq 0.001

590 **Figures**

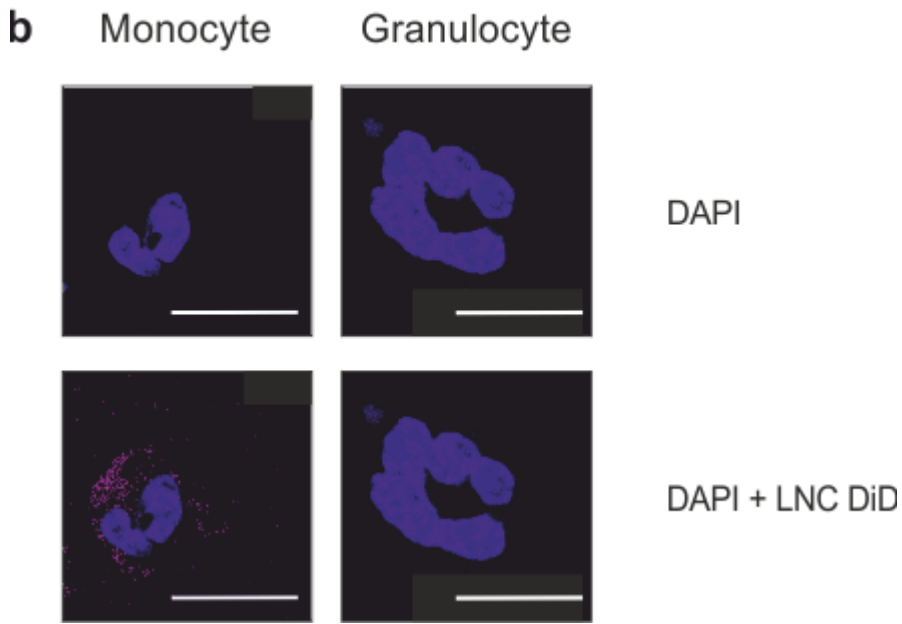
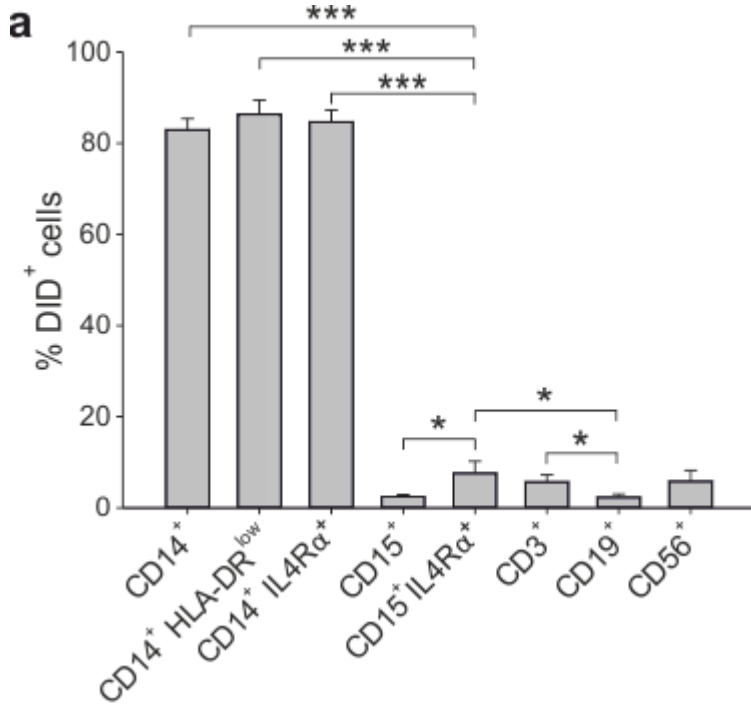
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591 **Fig.1**
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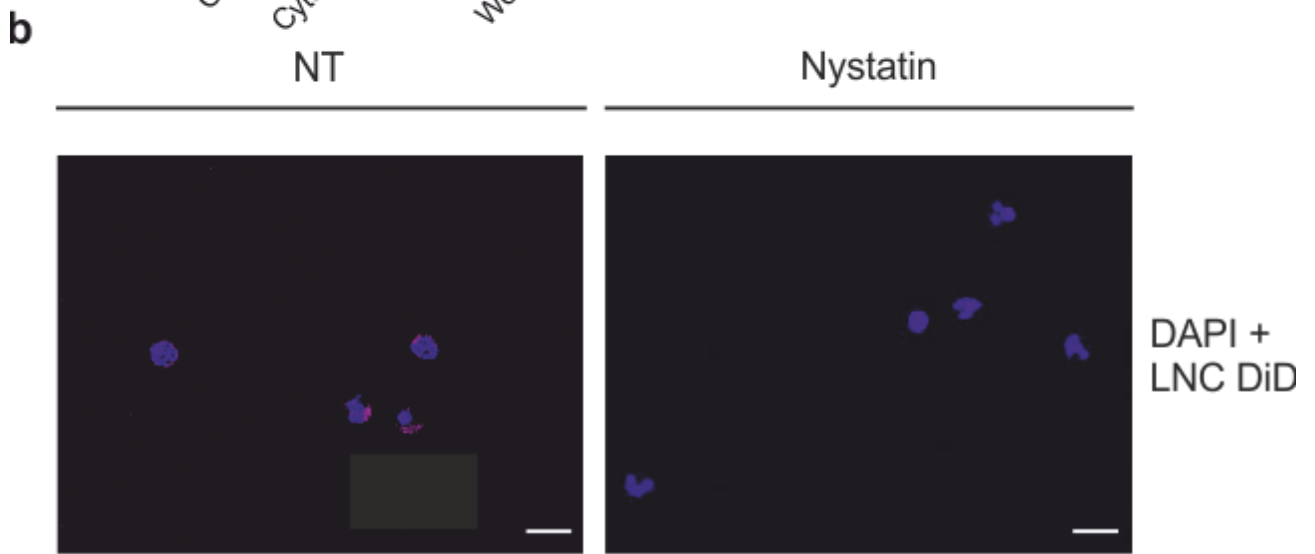
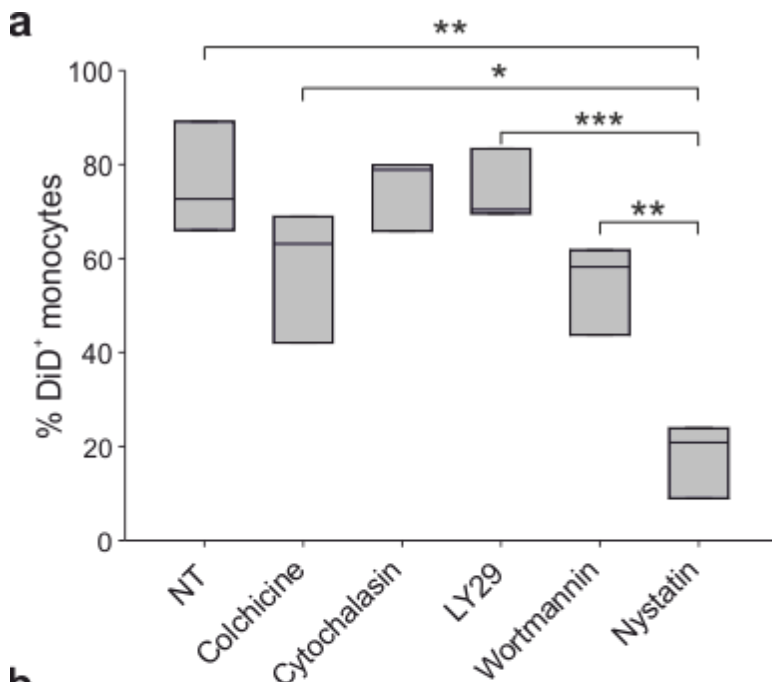
598 **Fig.2**



607 **Fig.3**



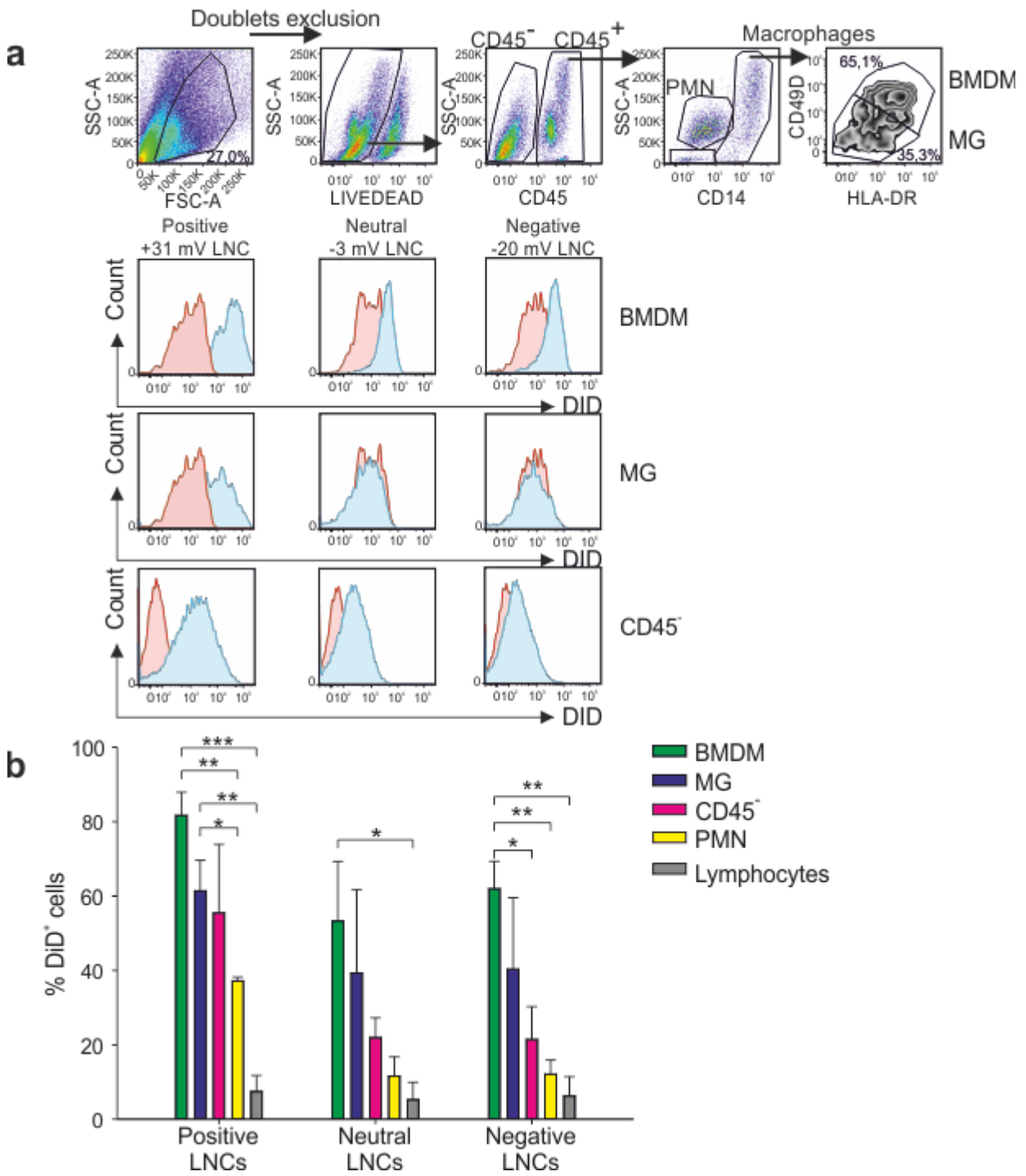
615 **Fig.4**



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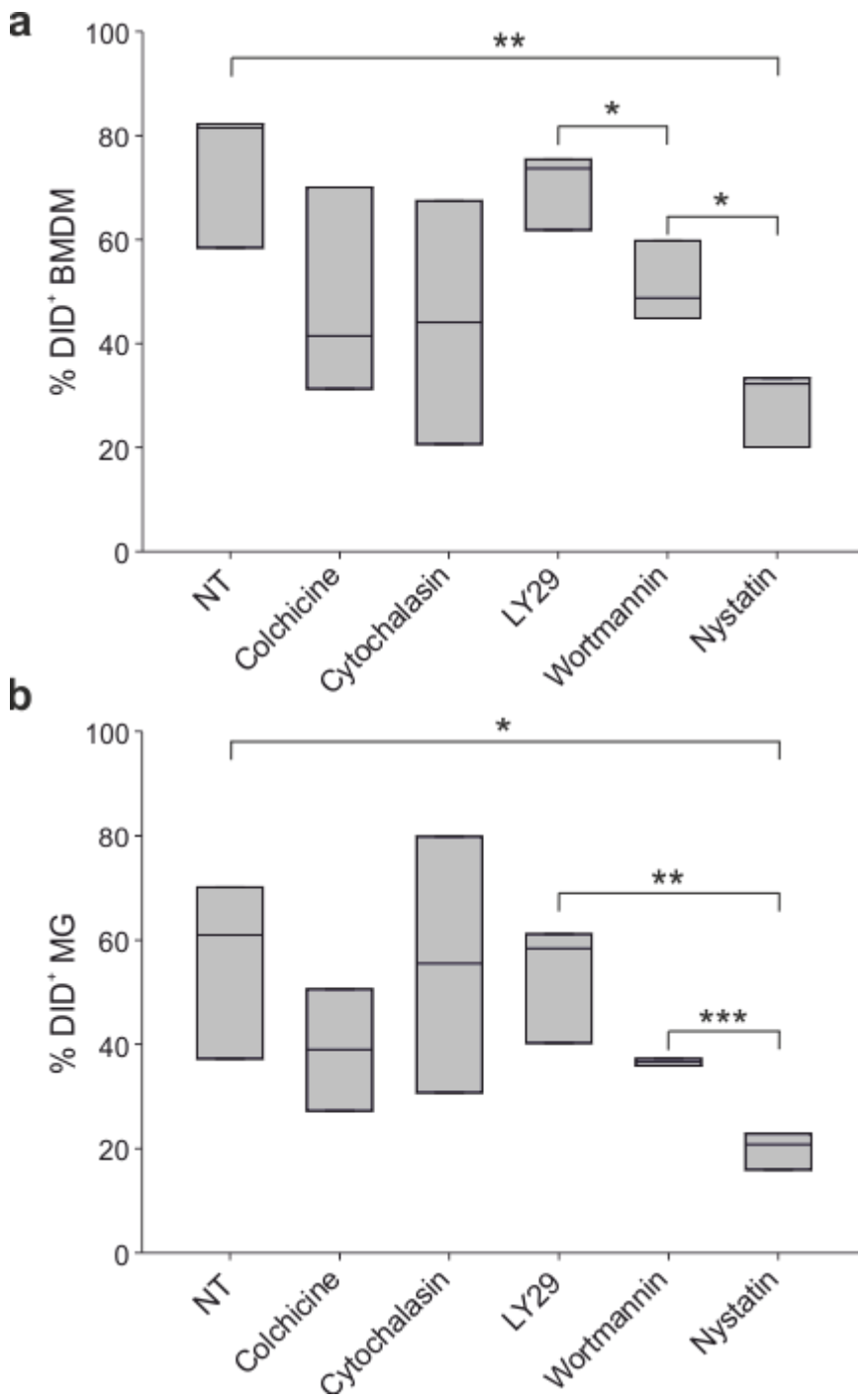
625 **Fig.5**

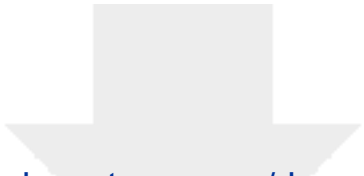
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632 **Fig.6**

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December 05, 2019

Dear Editors

Please find enclosed our manuscript entitled "Targeting of immunosuppressive myeloid cells from glioblastoma patients by modulation of size and surface charge of lipid nanocapsules" by Laura Pinton et al., to be considered for publication in "Journal of Nanobiotechnology" in the section "Research article".

In the last years, immune-mediated therapies have been recognized as powerful approaches to treat advanced cancers. While drug delivery nanosystems have been widely investigated to improve conventional cancer chemotherapy, their use to target immune cells in immune-mediated therapies is currently receiving growing attention by the scientific community.

We previously developed a formulation of lipid nanocapsules (LNC) loaded with a lauroyl-modified form of gemcitabine interacting preferentially with myeloid suppressive cells in mouse tumor models and in the blood of melanoma patients, thus setting the ground for a promising application of this formulation in cancer immunotherapy. In this manuscript, we extended these findings, and optimized the physico-chemical properties of LNC, in order to improve their targeting to myeloid cells from glioblastoma patients. We chose to focus on glioblastoma because we and others have demonstrated that myeloid cells orchestrate a strong immune suppression in this tumor. Candidate LNC formulations were then tested in the blood and tumor specimens of patients, to establish their incorporation by the different leukocyte subsets or by tumor cells. Our findings indicate that it is possible to obtain a very high incorporation of LNC by immune suppressive myeloid cell subsets, while sparing T lymphocytes and other immune cells, which play a positive role in controlling tumor growth and/or are required for the maintenance of normal host immune functions. By effectively targeting an immunosuppressive cell population, optimized LNCs may act to provide an efficient immunomodulatory activity, and enhance the therapeutic efficacy in combination therapies.

I believe that the findings presented in our manuscript will be of help to understand the interactions between delivery nanosystems and immune cells and will bring significant innovation for the application of nanotechnology to immunology and cancer therapy.

Thank you for receiving our manuscript and considering it for review. We appreciate your time and look forward to hearing from you.

Yours sincerely,

A handwritten signature in black ink, appearing to read "Susanna Mandruzzato". The signature is written in a cursive style with some flourishes.

Susanna Mandruzzato, PhD

