Rab27a supports exosome-dependent and –independent mechanisms that modify the tumor microenvironment and can promote tumor progression

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Abstract

During progression from single cancer cells to a tumor mass and metastases, tumor cells send signals which can subvert their tissue microenvironment. These signals involve soluble molecules and various extracellular vesicles, including a particular type termed exosomes. The specific roles of exosomes secreted in the tumor microenvironment, however, is unclear. The small GTPases RAB27A and RAB27B regulate exocytosis of multivesicular endosomes, which leads to exosome secretion, in human HeLa cells. Here, we used mouse models to demonstrate that Rab27a blockade in mammary carcinoma cells decreased secretion of exosomes characterized by endocytic markers, but also of matrix metalloproteinase MMP9, which is not associated with exosomes. Rab27a blockade resulted in decreased primary tumor growth and lung dissemination of a metastatic carcinoma (4T1), but not of a non-metastatic carcinoma (TS/A). Local growth of 4T1 tumors required mobilization of a population of neutrophil immune cells induced by Rab27a-dependent secretion of exosomes together with a specific combination of cytokines and/or metalloproteinases. Our findings offer in vivo validation of the concept that exosome secretion can exert key pathophysiologic roles during tumor formation and progression, but they also highlight the idiosyncratic character of the tumor context.

Abbreviations : Conditioned Medium, CM; Matrix MetalloProteinases, MMP

Introduction

Cell-autonomous acquisition of new properties such as proliferation and resistance to programmed death is not sufficient for a cell to become a tumor (1). Cell interactions within the microenvironment are now recognized as a crucial element in progression from single tumor cells to a local tumor mass and eventually distant metastases. Transformed cells exchange signals with surrounding fibroblasts, endothelial cells and immune cells both through direct cell-cell interactions, and through secreted molecules. For instance, tumors can secrete growth factors for endothelial cells, or chemokines and cytokines attracting and modifying the functions of immune cells. In addition, cells also secrete vesicles, i.e. membrane-enclosed structures (2-4), which expose transmembrane receptors, and contain proteins and RNA from the secreting cells. Vesicle interaction with surrounding cells can lead to activation of cell surface receptor, but also intracellular delivery of the vesicles' content (5, 6), thus ensuring modification of the target cells.

Various types of membrane vesicles are released by cells, and a subpopulation called exosomes has began receiving extensive attention in the past 4 years (7). Exosomes are small vesicles (50-100 nm in diameter) formed intracellularly in endocytic multivesicular compartments, and are released upon fusion of these compartments with the plasma membrane. Other vesicles, more heterogeneous in size (50 to 1000 nm), can be released from the cell surface, by a budding process similar to that used by some viruses. Exosomes are secreted by most cell types, including tumor and immune cells. Contradictory functions of tumor exosomes have been reported in vitro. On one hand, exosomes contain and transfer tumor antigens to dendritic cells for presentation of these antigens to T lymphocytes (8, 9), but on the other hand, they display inhibitory effects on effector immune responses (10, 11), and they have recently been proposed to promote metastasis (12). The resulting function(s) of exosome secretion by tumor cells in vivo thus remains unclear.

We have recently shown that the small GTPases RAB27A and RAB27B are critically required for exosome secretion by HeLa cells (13). RAB27A/B are known to control intracellular trafficking and regulated secretion of lysosome-related organelles (14, 15). In HeLa cells, we showed that spontaneous secretion of exosomes from CD63-containing compartments was strongly decreased if expression of either RAB27A or RAB27B was knocked-down by shRNA, whereas secretion of a soluble protein through the constitutive secretion pathway was not affected. We thus knocked-down Rab27a/b in mouse tumor cells to address the physiological functions of exosome secretion in vivo.

Our results show that, in two mammary carcinoma cells, 4T1 and TS/A, Rab27a is required for exosome secretion, whereas Rab27b is not. In vivo, growth and metastasis of 4T1 is impaired by Rab27a inhibition, which prevents systemic mobilization of a pro-tumoral population of neutrophils, whereas for TS/A, neither local growth nor formation of metastasis (which is very limited for this cell line) are affected. Rab27a is also involved in the secretion of some non exosome-associated proteins by the two cell lines, especially the pro-metastatic matrix metalloprotease (MMP) 9. Finally, we show complementary effects of exosomes, soluble cytokines and/or metalloproteases in modulation of the immune system by the growing tumors: a different quantitative and qualitative secretion of both exosomes and soluble proteins by 4T1 and TS/A explains their different dependency to Rab27a. In conclusion, our results highlight a pro-tumoral function of Rab27a expression, mediated by both exosome-dependent and –independent secretions, in some, but not all, tumors.

Materials and Methods

Mice

Balb/c female mice were obtained from Charles Rivers France. Balb/c $Rag2^{-/-}\gamma c^{-/-}$ mice and corresponding WT controls were obtained from E. Vivier (CIML, Marseille, France). Mice were housed in specific pathogen-free conditions and experiments were done in accordance with the guidelines of the French Veterinary Department.

Cells

4T1 was obtained from S. Fiorentino (Columbia, ATCC origin) and TS/A from L. Zitvogel (Institut Gustave Roussy, Villejuif, France). Absence of mycoplasma contamination was checked monthly. Cells were authenticated by their ability to grow and metastasize in immunocompetent hosts as described in the literature. For stable inhibition of Rab27a or Rab27b expression, cells infected with shRNA-expressing lentiviruses were selected and maintained in medium containing 5 μg/mL puromycin (Invitrogen). Cells were used within one month after lentivirus infection. Independent experiments were performed with batches of independently infected cells.

Reagents

Detailed list of antibodies for FACS and Western Blotting is given as supplemental materials. Mouse Anti-Rab27a was generated in our lab (M. Seabra (14)). pLKO.1puro plasmids allowing expression of shRNA specific for mouse Rab27a or Rab27b, or a scrambled sequence of shRNA to GFP as control (Scr), and a puromycin resistance gene were obtained by L.F.Moita from the library described in (16).

Exosome purification and characterization

Exosome purification was performed as previously described by differential ultracentrifugation (17), from 48 hours-conditioned medium (CM) generated in complete medium depleted from calf serum-derived exosomes. Proteins were quantified by Micro-BCA

(Thermo Scientific) with 2% SDS. Exosomes secreted by 10-15x10^6 cells (<9 µg) or 30 µg of lysates (2-3x10^5 cells) were loaded on NuPAGE 4-12% BisTris gels (Invitrogen) and separated under non-reducing conditions (except for Rab27a analysis: reducing conditions, 150 µg of lysates).

Quantitative RT-PCR

Quantitative RT-PCR was performed using Absolute Q-PCR SYBRGreenROX Mix (Abgene) on a Lightcycler LC480 (Roche). Primers were purchased from Qiagen (QuantiTect Primer Assay). Cycle threshold (Ct) for *Rab27a* and *Rab27b* were normalized to Ct for *Gapdh* and results were expressed either as arbitrary units (AU: $2^{Ct(GAPDH)-Ct(gene)}$x1000) or calculated as percentage of control shRNA-transduced cells.

In vivo tumor growth and metastasis development

Mice were injected subcutaneously in the mammary fat pad region with 50,000 cells, or intravenously with 100,000 cells. Tumor volume (= length x width x [(length + width)/2]) was measured twice weekly. Mice were sacrificed when tumors reached 1,500 mm^3, or 20 days after intravenous injection. Lungs were fixed in AFA and nodules were manually counted. Anti-Ly6G antibodies were injected everyday i.p. at 50 (d3) and 25 µg/mouse (d4-d15), and twice a day at 50 µg/mouse (d16-d18). Scr-4T1 or Scr-TS/A exosomes were injected into tumors in 50 µl of PBS at 1, 2, and 5 µg/tumor (day 3, 6, and 9 onwards respectively).

Analysis of immune cells by flow cytometry

Single cell suspensions from organs or from bone marrow cultures stained with mixed fluorescent antibodies were acquired on a MacsQuant (Miltenyi Biotech). Analyses were performed with FlowJo software.

Analysis of secreted factors

Soluble secreted factors were analyzed in CM before exosome purification. CM depleted or not of exosomes (by 1h ultracentrifugation at 100,000 g), cell lysates and exosomes (in 0.5%

Triton X-100) were used to measure cytokines and MMPs by ELISA (R&D systems or Raybiotech), CBA (BDBiosciences) or FlowCytomix (eBiosciences). Concentrations of cytokines were rapported to the number of producing cells.

Bone marrow differentiation in vitro

Briefly, Balb/c bone marrow cells plated in non culture-treated 24-well plates with tumor cell CM were cultured for seven days with addition of fresh medium every two days. CM from $3x10^5$ or $1x10^5$ cells was added to $3.7x10^5$ bone marrow cells. To assess the relative contribution of soluble and particulate (exosomal) components, CM was sequentially ultracentrifuged up to 100,000 g, to generate exosomes (pellet) and exosome-depleted CM (supernatant). On day 7, pooled floating and adherent cells were analyzed by FACS on a MacsQuant flow cytometer, set to allow quantification of absolute cell number.

Results

Differential expression of Rab27a and Rab27b in mouse tumor cells and efficient inhibition by shRNA

We first analyzed the expression of *Rab27a* and *Rab27b* in six mouse tumor cells of different tissue origins. *Rab27a* mRNA was strongly expressed in the melanoma B16F10, whereas *Rab27b* was undetectable (Figure 1A). By contrast, *Rab27b* was readily detected in a bladder carcinoma, MB49, where Rab27a expression was weak. The fibrosarcoma MCA101 expressed both *Rab27a* and *Rab27b* at low levels, and two mammary carcinomas, TS/A and 4T1, expressed both genes at equivalent and readily detectable levels. Analysis of Rab27a expression at the protein level confirmed the mRNA results (Figure 1B).

As previously done in the HeLa cell line, which expresses both genes at equivalent levels ((13)), we used lentiviruses expressing shRNA to mouse *Rab27a* or *Rab27b*, or a control non-murine gene (Scr), to infect the two mammary carcinoma cell lines. Among five shRNA sequences specific for *Rab27a* (respectively *Rab27b*), only sh27a2 (respectively sh27b1) induced significant downregulation of *Rab27a* without major alteration of expression of *Rab27b* (respectively *Rab27a*), in both TS/A and 4T1 (supplemental Figure S1, Figure 1C). Western blot analysis confirmed a strong decrease of Rab27a protein in the sh27a2-expressing cells, with no significant modification in the sh27b1-expressing ones (Figure 1D).

Inhibition of Rab27a, but not Rab27b, affects exosome secretion

Exosomes were purified by differential ultracentrifugation from conditioned culture medium (CM) (17) of cells expressing stably shRNA 27a2 and 27b1. The total amount of proteins recovered in the 100,000 g pellet, corresponding to the smallest membrane vesicles including exosomes, was twice lower per secreting cell in TS/A than 4T1 (Figure 2A). We observed a consistent reduction of this amount in both TS/A and 4T1 cells expressing sh27a (Figure 2A).

By contrast, cells expressing sh27b secreted variable amounts of exosomal proteins, not significantly different from what was released by control cells. Analysis of four different exosome markers (CD63, Tsg101, Alix, Hsc70) in the secreted vesicles showed a significant decrease of secretion of all of them in sh27a-expressing 4T1 and TS/A, but not in sh27b-expressing cells (Figure 2B). Thus, Rab27a is required for secretion of vesicles bearing endosomal markers (CD63, Alix, Tsg101), i.e. corresponding to the original definition of exosomes. Rab27a therefore plays a similar role in the two mouse mammary carcinoma cells and in the human cervical carcinoma cell HeLa (13). Rab27b, by contrast, is not consistently required for exosome secretion by 4T1 and TS/A, whereas it is in HeLa cells (13). Different cells can thus use differently the intracellular machinery for exosome secretion. In addition, we recently showed that secretion of other vesicles bearing non-endosome markers (Mfge8 and CD9) is not affected by Rab27a inhibition (18).

Inhibition of Rab27a impairs 4T1 but not TS/A tumor growth in vivo

Since exosomes from either 4T1 or TS/A have been shown to promote tumor growth when injected in vivo (19, 20), we then asked whether Rab27a inhibition in tumor cells would change growth of these tumors in vivo. After subcutaneous injection in syngeneic Balb/c hosts, growth of sh27a-TS/A tumors was identical to growth of their control (Scr) counterparts (Figure 3A). By contrast, Rab27a-impaired 4T1 tumors grew significantly more slowly than control (Scr) 4T1 cells (Figure 3B), and induced lower number of lung metastases (Figure 3C). Lower incidence of metastases was also observed after i.v. injection of the sh27a-4T1 tumors (Supplementary Figure S2A), showing that decreased metastatic ability of the locally growing sh27a-tumors was not simply due to smaller size of the subcutaneous tumors, but also to reduced ability to colonize lungs. The difference in in vivo growth was not

due to intrinsic slower cell proliferation, since sh27a- and Scr-4T1 cells grew with identical rates in vitro (supplemental Figure S2B).

4T1 modulates the immune system in a Rab27a-dependent manner

We thus hypothesized that Scr and sh27a-tumors modulated differently their microenvironment, and we focused on its immunological side. Analysis of immune cell populations in tumor-bearing mice showed strong infiltration of Scr-4T1 tumors by cells co-expressing the markers CD11b, Ly6C and Ly6G (up to 70% of the immune cells, Figure 4A, supplementary Figure S3A) displaying multi-lobed nucleus and clear cytoplasm, typical of neutrophils. This population was less abundant in sh27a-4T1 tumors, where it represented at most 25% of immune cells, regardless of the respective sizes of sh27a- and Scr-4T1 tumors (Figure 4A middle panel). Other immune cells were concomitantly upregulated in sh27a-4T1 tumors, but the difference was statistically significant only for CD4+ T lymphocytes (Figure 4B). Neutrophils were also significantly more abundant in the spleen (Figure 4A) and blood (not shown) of Scr-4T1 as compared to tumor-free or sh27a-4T1-bearing mice. Thus Rab27a is required for 4T1's capacity to modulate systemically the immune system of host mice. Immune populations infiltrating TS/A tumors were very different from those infiltrating 4T1, with low amounts of neutrophils (maximum 12% of the immune infiltrate), and comparatively more CD4+ T and NK lymphocytes (Figure 4A, B). No differences were observed between Scr and sh27a-TS/A tumors.

To determine whether immune cells participate in differential growth of Scr- and sh27a-4T1, tumors were grown in hosts devoid of B and/or T lymphocytes ($Rag2^{-/-}$ hosts or depletion of CD4 T cells, Supplementary Figure S3B-C), of all adaptive and NK cells ($Rag2^{-/-}\gamma c^{-/-}$ hosts, Figure 4C), or of neutrophils (depletion by anti-Ly6G antibody, Figure 4D). Both Scr- and sh27a-4T1 grew more efficiently in $Rag2^{-/-}\gamma c^{-/-}$ than WT hosts, showing that Rab27a-impaired

tumors are not intrinsically unable to grow in vivo. But sh27a-4T1 still grew significantly less efficiently than Scr-4T1 in the absence of T, B and/or NK lymphocytes (Figure 4C, supplementary Figure S3B-C), thus showing that the pro-tumoral effect of Rab27a does not involve these immune cells. By contrast, inhibition of neutrophil invasion strongly impaired growth of Scr-4T1 tumor, which grew similarly as neutrophil-poor sh27a-4T1 (Figure 4D). Therefore, the 4T1 tumor has developed a Rab27a-dependent capacity to modulate the host's immune system, namely neutrophils, to its own advantage, whereas TS/A does not rely on such immune system-dependent mechanism to grow.

Inhibition of Rab27a affects secretion of some non exosome-associated proteins

We then asked whether secretion of non-exosome associated molecules could be also affected upon Rab27a inhibition. Secretion of 144 soluble or membrane-associated proteins was measured by antibody microarrays in CM of cultured cells (supplemental Figure S4). Twenty-three proteins were secreted above the background level, with differences in the secretomes of 4T1 and TS/A, especially extracellular proteases (pro-MMP9, MMP2 and MMP3), and cytokines and chemokines modulating myeloid cells and neutrophils (G-CSF/Csf3, MCP-1/Ccl2, RANTES/Ccl5) (21, 22). Interestingly, Rab27a inhibition seemed to change the level of secretion of a subset of these proteins.

We thus used quantitative assays to measure secretion of the most relevant proteins by 4T1 and TS/A upon inhibition of Rab27a. We confirmed that 4T1 secretes high levels of pro-MMP9 and MMP3, whereas TS/A secretes high level of MMP2 and some pro-MMP9. Rab27a inhibition abolished secretion of pro-MMP9 in both cells, without affecting secretion of either MMP3 or MMP2 (Figure 5A). Since secretion of some MMPs in association with membrane vesicles including exosomes has been described (23, 24), reduced secretion of MMP9 in Rab27a-impaired cells could be due to reduced exosome secretion. However, by

comparing the amount of MMP9 present in CM from 10^5 cells, with the same CM after successive ultracentrifugation to deplete exosomes, or with exosomes obtained from 5.10^6 cells, we showed that the vast majority of MMP9 is secreted by 4T1 (Figure 5B) and TS/A (not shown) as a soluble form, rather than associated with exosomes. As a control, we confirmed that the vesicle-associated protein Mfge8 (25) was detected in these conditions at comparable levels in exosomes (Exo) and the CM (Figure 5B), where its amount was decreased by at least 30% after exosome depletion. Thus Rab27a inhibition independently impairs exosome and MMP9 secretion.

The effects of Rab27a inhibition on cytokine and chemokine secretions were also contrasted. 4T1 secretes at higher level than TS/A the neutrophil-specific growth factor G-CSF, and at lower levels the myeloid cell chemotactic factors MCP-1 and RANTES (Figure 5C). Upon Rab27a inhibition, both cells displayed increased secretion of G-CSF, and tendencies to increased secretion of MCP-1, and decreased secretion of RANTES. Like for MMP9, none were secreted in association with vesicles (Figure 5D).

Our results thus show that 4T1 and TS/A display very different secretomes, and that Rab27a, in addition to promoting secretion of endosome-derived exosomes, also regulates secretion of a subset of soluble proteins in these cells. Since G-CSF is the canonical growth factor for granulocytes, its secretion at high level by 4T1 probably explains the accumulation of neutrophils observed in vivo (Figure 4). But impaired accumulation of neutrophils in sh27a-4T1 tumors cannot be explained by inhibition of G-CSF secretion, since this tumor secretes even more G-CSF.

Secreted soluble and pelletable factors cooperate to promote accumulation of neutrophils.

To determine whether exosomes could be responsible for the diverse patterns of immune cells observed in mice bearing the different tumors, we first analyzed in vitro the effect of CM from 4T1 and TS/A on survival and differenciation of bone marrow hematopoietic cells (Figure 6). After 7 days, cells cultured with CM of Scr-4T1 or sh27a-4T1 were more abundant than those grown in unconditioned medium or CM of TS/A (Figure 6A). In addition, ultracentrifugation of the CM at 100,000 g did not decrease the survival effect (data not shown). Thus, soluble cytokines secreted by 4T1 (probably G-CSF) promote bone marrow cell survival, and/or proliferation in vitro.

Analysis of the cell types present in these cultures showed that the CM of 4T1 promoted CD11b+/Ly6C+/G- and Ly6C+/G+ cells (Figure 6B). The CM of TS/A, by contrast, promoted CD11b+/Ly6C-/G-, and CD11c+/I-Ad+ dendritic cells. CM of sh27a-4T1 cells or ultracentifuged CM of Scr-4T1 were both less efficient than the CM of Scr-4T1 at promoting Ly6C+/G+ neutrophils (Figure 6C, CM), and more efficient at promoting Ly6C+/G- cells (Figure 6B). Complementation of the ultracentrifuged CM with its 100,000 g pellet (Figure 6C, depl-CM+Exo) reconstituted the initial activity. Therefore, in a cytokine environment promoting survival of non-dendritic myeloid cells, such as the one generated by 4T1, exosomes secreted in a Rab27a-dependent manner specifically promote survival and/or differenciation of neutrophils.

To test whether this applied to in vivo growth of tumors, we injected exosomes purified from Scr-4T1 cells into growing sh27a-4T1 tumors. This treatment allowed increased growth of sh27a-4T1 (Figure 6D), and also increased systemic accumulation of neutrophils in spleen of tumor-bearing mice (Figure 6E), whereas injection of exosomes purified from Scr-TS/A did not induce either growth or neutrophil mobilization. Interestingly, neutrophil accumulation was not observed in sh27a-4T1 tumors themselves after exosome injection, where instead, both 4T1- and TS/A-exosomes induced accumulation of Ly6C-/G- myeloid cells (Figure 6E).

Thus exosomes secreted by 4T1 display a specific ability to facilitate local tumor growth, at least partly by inducing systemic mobilization of neutrophils.

Discussion

The work described here demonstrates a tumor-promoting role of Rab27a expression by a mouse metastatic breast carcinoma, mediated by modulating the tumor immune microenvironment through secretion of cytokines and of exosomes, as well as the proteolytic environment through secretion of MMP9.

We had previously shown in HeLa cells that RAB27A and RAB27B were required for efficient secretion of exosomes but not of a protein secreted through the regular secretion pathway (13). Here, in two murine tumor models, Rab27a is required for secretion of exosomes, but also of non exosome-associated MMP9, whereas it inhibits secretion of a subset of cytokines. Similar observations have recently been published on the B16F10 melanoma cell line, where Rab27a shRNA decreased secretion of exosomes and also of some angiogenic growth factors (PlGF, PDGF), while increasing secretion of protease inhibitors (TIMP1) (12). Complex roles of Rab27a in regulated secretion have been described in various secretory cells (26-29). Further studies will be required to determine the molecular mechanisms responsible for Rab27a-regulated expression and/or secretion by tumor cells of some cytokines and we are currently performing such analyses for proteases (C. Recchi, M. Seabra et al, in preparation). In any case, our observations show that Rab27a inhibition does not affect exlusively exosome secretion, and thus its use to understand the functions of exosomes in vivo must be completed, as we did here, with experiments to distinguish the relative contribution of cytokines and vesicles in the phenotypes observed. Furthermore, we observed that Rab27b inhibition did not affect the secretion of exosomes by the two murine carcinomas as it did in HeLa cells (13). Thus Rab27 proteins are not universal regulators of exosome secretion, and our results highlight the diversity of intracellular compartments and of the molecular machineries used by different cell types for trafficking and fusion of these compartments.

The second important observation reported here is that Rab27a inhibition modulates differently in vivo growth of two tumor cell lines of the same tissue origin. 4T1 (30) and TS/A (31) are two mammary adenocarcinomas, but the latter has been described as more immunogenic than the former (32), and in our hands it was less metastatic. We chose these cells to study the physiological functions of in vivo secreted exosomes, because both had been shown to secrete in vitro exosomes with immunosuppressive potential (19, 20): inhibiting NK cell activity in vivo, inducing accumulation in spleen of CD11b+/Gr1+ cells (the anti-Gr1 antibody labels Ly6C and Ly6G), preventing proper differenciation of dendritic cells induced by GM-CSF in vitro (19), and promoting expression of myeloid suppressor cell genes (33, 34). Another group also showed that exosomes from various mouse tumor cell lines, including TS/A, promoted the differenciation in vitro of CD11b+/Gr1+ cells with suppressor effects on adaptive immune responses (35). In our work, by inhibiting Rab27a gene expression, we decreased secretion of exosomes by more than 50% in the two cell lines, but this led to decreased tumor growth in vivo of only one of them. Based on our in vivo and in vitro data, we propose that soluble cytokines and exosomes secreted in vivo by 4T1 enter the blood circulation and reach the bone marrow (as shown before for injected exosomes (19)). There, cytokines promote proliferation of granulocyte precursors, whereas exosomes orient their differenciation into neutrophils. These cells will then leave the bone marrow to reach lymphoid organs and eventually the tumor. In this context, TS/A does not secrete the cytokines necessary to promote granulocyte differenciation, and its exosomes are either not efficient at orienting granulocyte differenciation or not able to leave the tumor to reach myeloid precursors. Identifying the exosomal components responsible for the observed functional difference in TS/A and 4T1 will be the subject of future work.

The way immune cells mobilized by 4T1 participate in tumor progression is also not fully determined in this manuscript. The CD11b+/Ly6C+/G+ markers used here classically define

neutrophils (and their morphology in cytospin confirms their neutrophilic nature, Figure 4A), but have also been used to characterize a population called « myeloid-derived suppressor cells », which inhibits T lymphocyte activation (36). NK cells and the adaptive immune system, however, are not required for the Rab27a-dependent tumor promoting activity of 4T1 (Figure 4E). The neutrophil population accumulating in 4T1-bearing mice is thus modifying the tumor microenvironment independently of the adaptive immune system, possibly by secreting factors promoting angiogenesis and vascular remodeling, or helping tumor cell migration, as shown by other groups (21, 37).

Finally, we showed abolition of pro-MMP9 secretion in Rab27a-impaired 4T1 cells. A strict requirement of MMP9 expression and secretion has been previously demonstrated for efficient metastasis of 4T1 (38), thus the pro-metastatic effect of Rab27a in 4T1 is probably due to its role in MMP9 secretion. TS/A, by contrast, secretes another gelatinase, MMP2, whose secretion is not affected by Rab27a inhibition, and whose role in degrading extracellular matrix and promoting invasion has been previously shown in human breast cancer cell lines (39). Our observation that two different mouse mammary carcinoma secrete completely different patterns of metalloproteases with different dependence on Rab27a thus suggests that the effect of Rab27a on tumor cell's ability to degrade the extracellular matrix, like the effect on the immune system, will vary from one cell to another.

In conclusion, our work conclusively demonstrates that local secretion of exosomes by a tumor in vivo can promote tumor progression, but also that it would be very dangerous to generalize such a mechanism to all tumors, and to propose that "tumor-derived exosomes promote tumor progression" whatever the tumor and the model studied. Keeping in mind the idiosyncrasy of each tumor is very important for researchers, but also for clinicians, and this idea is at the basis of the currently expanding trend of "personalized therapies" taking into account for treatment the specific characteristics of each individual and his/her tumor.

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Figure legends

Figure 1: Rab27a and Rab27b are differently expressed in mouse tumor cell lines, and shRNA can inhibit their expression in mammary tumor cells.

A- Expression of *Rab27a* and *Rab27b* by quantitative RT-PCR in mouse dendritic cells (DCs), and tumor cell lines: B16F10, MCA101 (MCA), TS/A, 4T1, MB49. mRNA level of *Rab27a* (left panel) or *Rab27b* (middle panel) is expressed in arbitrary units (A.U.) as compared to *Gapdh*, and the relative level of *Rab27a* and *Rab27b* is calculated by the ratio of A.U (right panel). mean + s.d. from 1 (MB49), 2 (4T1, B16F10), 3 (DC) or 4 (MCA, TS/A) experiments. **B-** Expression of Rab27a protein cell lysates from wild-type (DCwt) or Rab27a mutant (DCAsh) dendritic cells, and the tumor cell lines. Actin expression is shown as loading control. **C-** Expression of *Rab27a* and *Rab27b* mRNA in TS/A and 4T1 cells expressing shRNA to either Rab27a (sh27a=sh27a2) or Rab27b (sh27b=sh27b1) as compared to control shRNA (Scr). % of expression level in control cells, mean + s.d. from 6 (TS/A sh27b), 9 (TS/A sh27a and Scr), 13 (4T1 sh27b) or 14 (4T1 sh27a and Scr) experiments are shown. **D-** Expression of Rab27a protein TS/A or 4T1 expressing shRNA to either Rab27a (sh27a) or Rab27b (sh27b) as compared to control shRNA (Scr). Actin expression is shown as loading control.

Figure 2: Inhibition of Rab27a, but not of Rab27b, decreases secretion of exosomes.

A- Total amount of exosomal proteins obtained from control (Scr), Rab27a-impaired (sh27a) or Rab27b-impaired (sh27b) 4T1 (left panel) or TS/A cells (right panel), quantified in 3-4 independent experiments. * = $p<0.05$, paired student's t-test. **B, C-** Western blot characterization of exosomes (Exo) secreted by control (Scr), sh27a- or sh27b-expressing 4T1 and TS/A. Cell lysates (cell) were analysed in parallel. One representative Western blot (B) and quantification (C) in exosomes obtained from sh27-expressing cells, as compared to

control cells are shown (mean + s.d. of 4-7 independent experiments). * = $p<0.05$, ** = $p<0.01$, Anova with Dunnett's post test.

Figure 3: Local growth and metastases formation by 4T1 (but not by TS/A) is decreased by inhibition of Rab27a.

A, B- Control (Scr), or sh27a-expressing TS/A (A) or 4T1 (B) tumors were injected subcutaneously in the mammary area of syngeneic Balb/c mice. Tumor growth over 4 weeks (left panels), and tumor size at d15 and d25 (right panels) are represented for individual mice (pooled from 3-4 independent experiments). Growth of 4T1-sh27a tumors, but not of TS/A-sh27a, is significantly impaired (*** = $p<0.001$, n.s. = $p>0.05$, student's T-test). **C,** Lung metastases were manually counted 28 days after subcutaneous injection of Control (Scr) or sh27a-expressing 4T1 cells, or indirectly evaluated by weighting lungs. Ctl = non-tumor injected mice. Results from individual mice pooled from 4 experiments are shown. (* = $p<0.05$, ** =$p<0.01$. One way ANOVA, Dunnett's post test.)

Figure 4: Rab27a inhibition prevents systemic accumulation of tumor-promoting neutrophils in mice bearing 4T1 tumors.

A- Quantification of CD11b+/Ly6C+/G+ cells (inset = typical neutrophil aspect in cytospin, scale bar = 10 µm) in individual tumors (left panel) or spleen (right panel) of mice bearing Scr and sh27a-4T1 or TS/A at d13-15 after implantation (left panel), and as a function of tumor size in 4T1 (middle panel). **B-** Quantification of other hematopoietic cells in the same tumors: CD11b+/Ly6C+/G- = macrophages and monocytes, CD4+ (Foxp3-) = conventional CD4 T lymphocytes, NKp46 = NK cells. *** = $p< 0.001$, ** = $p< 0.01$, * = $p<0.05$. One way ANOVA with Bonferronni's post test. **C-** Growth of Control (Scr) and sh27a-expressing 4T1 tumors in $Rag2^{-/-}/\gamma c^{-/-}$ as compared to wild-type (WT) Balb/c mice. Tumor size at d15 is

represented. Absence of T, B and NK lymphocytes does not abolish impaired sh27a-4T1 growth. **D-** Growth of Control (Scr) and sh27a-expressing 4T1 tumors in Balb/c mice depleted from neutrophils (anti-Ly6G), as compared to control antibody-injected mice (isotype). Tumor size at d15 (left panel) and mobilization of neutrophils in tumors (right panel) are represented. Growth of Scr-4T1 tumor becomes similar to growth of sh27a-4T1 upon neutrophil depletion (* = $p<0.05$, *** = $p<0.001$, student's T-test). Results from individual mice pooled from 2-3 independent experiments are shown.

Figure 5: 4T1 and TS/A secrete different patterns of non-exosome bound proteins, some of which are modified upon invalidation of Rab27a.

Quantification of proteins in conditioned medium (CM) from Scr or sh27a-expressing 4T1 (upper panels) and TS/A (lower panels). Individual results from 3-4 independent experiments are shown. * = $p<0.05$, ** = $p<0.01$, student's T-test. **A-** ELISA for pro-MMP9, MMP3 and MMP2. **B**- Quantification of pro-MMP9 and Mfge8 in total cell lysates (Cells), conditioned medium (CM), CM after 100,000 g ultracentrifugation (CM-E), and 100,000 g pellets (Exo) from 4T1 cells. **C, D-** ELISA for RANTES, MCP-1 and G-CSF in CM (C) and after ultracentrifugation as above (D). Results are expressed in pg / 10^5 (cells, CM, CM-E) or pg / 5.10^6 cells (Exo). n.d. = not detectable.

Figure 6 : Soluble and particulate factors secreted by 4T1 affect in vitro survival and differenciation of bone marrow-derived neutrophils.

A-C- Bone marrow cells were cultured for 7 days in the presence of non-conditioned medium (Medium) or CM from Scr- or sh27a- expressing 4T1 or TS/A (two dilutions, corresponding to CM of 1-3.10^5 cells). **A-** Number of live cells. Mean + s.d in one representative experiment out of 3. **B-** Nature of the cells (average % + s.e.m from 3 experiments): non

myeloid cells (CD11b-/CD11c-); dendritic cells (CD11c+/I-Ad+); monocytes and NK cells (CD11b+/Ly6C-/G-); macrophages and inflammatory monocytes (CD11b+/Ly6C+/G-); neutrophils (CD11b+/Ly6C+/G+). **C-** Percentage of neutrophils after culture in CM from Scr- or sh27a-4T1 before (total CM) or after (depl-CM) ultracentrifugation at 100,000 g, or in depl-CM reconstituted with its 100,000 g pellet (depl-CM + Exo). sh27a-expressing 4T1 are less efficient than Scr-4T1 at promoting neutrophils in vitro, and pelletable factors secreted by Scr-4T1 are responsible for this difference. (mean + s.d of 2 experiments). paired student's T-test * = $p<0.05$, ** = $p<0.01$, n.s. = non significant. **D, E-** Growth (D, tumor size at d19) and percent myeloid cells in spleens (E, left panel) and tumor (E, right panels) of Scr-4T1 or sh27a-4T1 after intratumoral injection of exosomes. Results from individual mice in 1 experiment are shown. ** = $p< 0.01$, * = $p<0.05$. One way ANOVA with Bonferronni's post test. Exosomes from 4T1, but not from TS/A reconstitute growth and increase systemic mobilization of neutrophils in sh27a-4T1-bearing mice. Both exosomes induce accumulation of Ly6C-/G- cells in the tumor.

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FIGURE 1

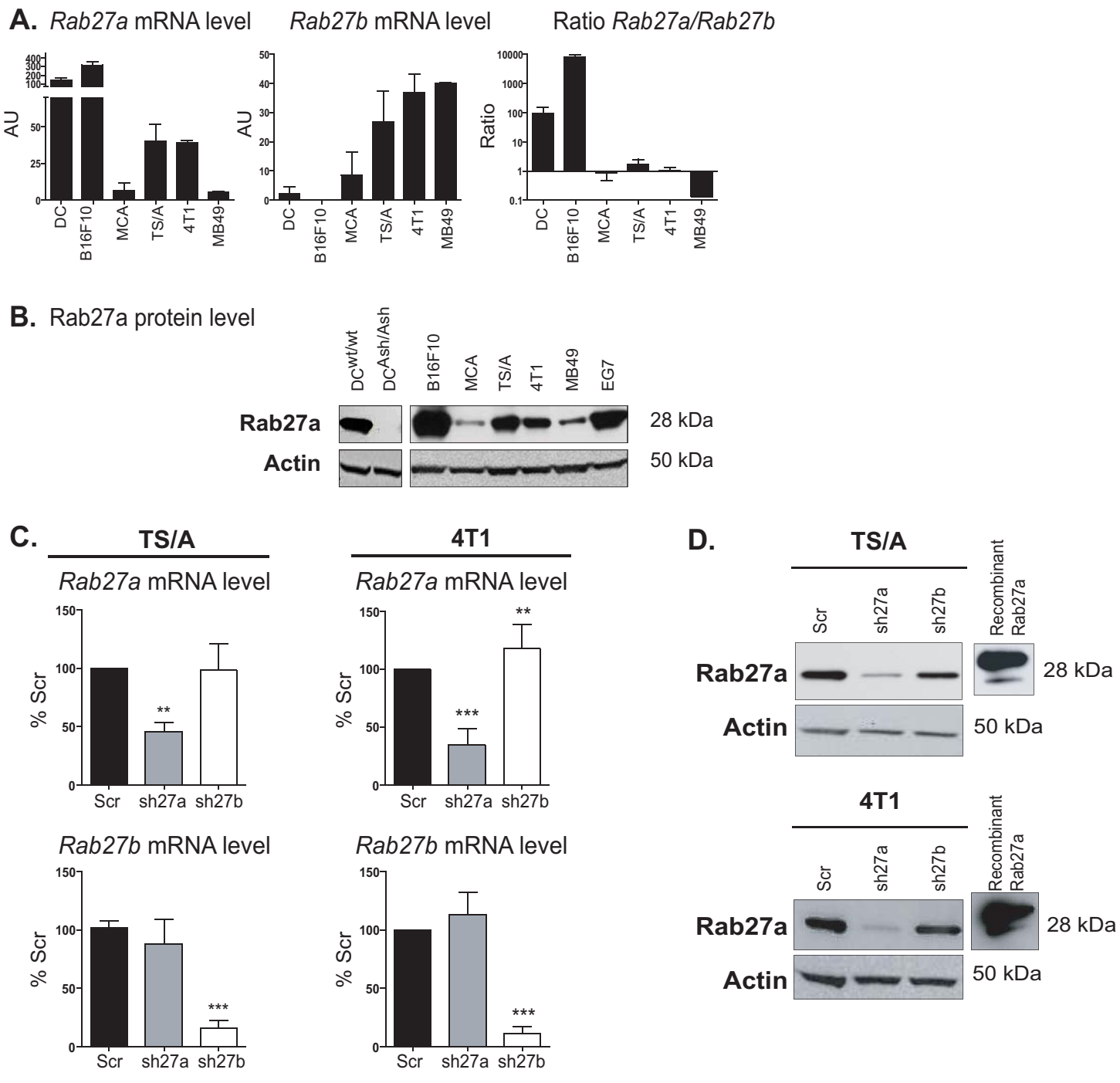
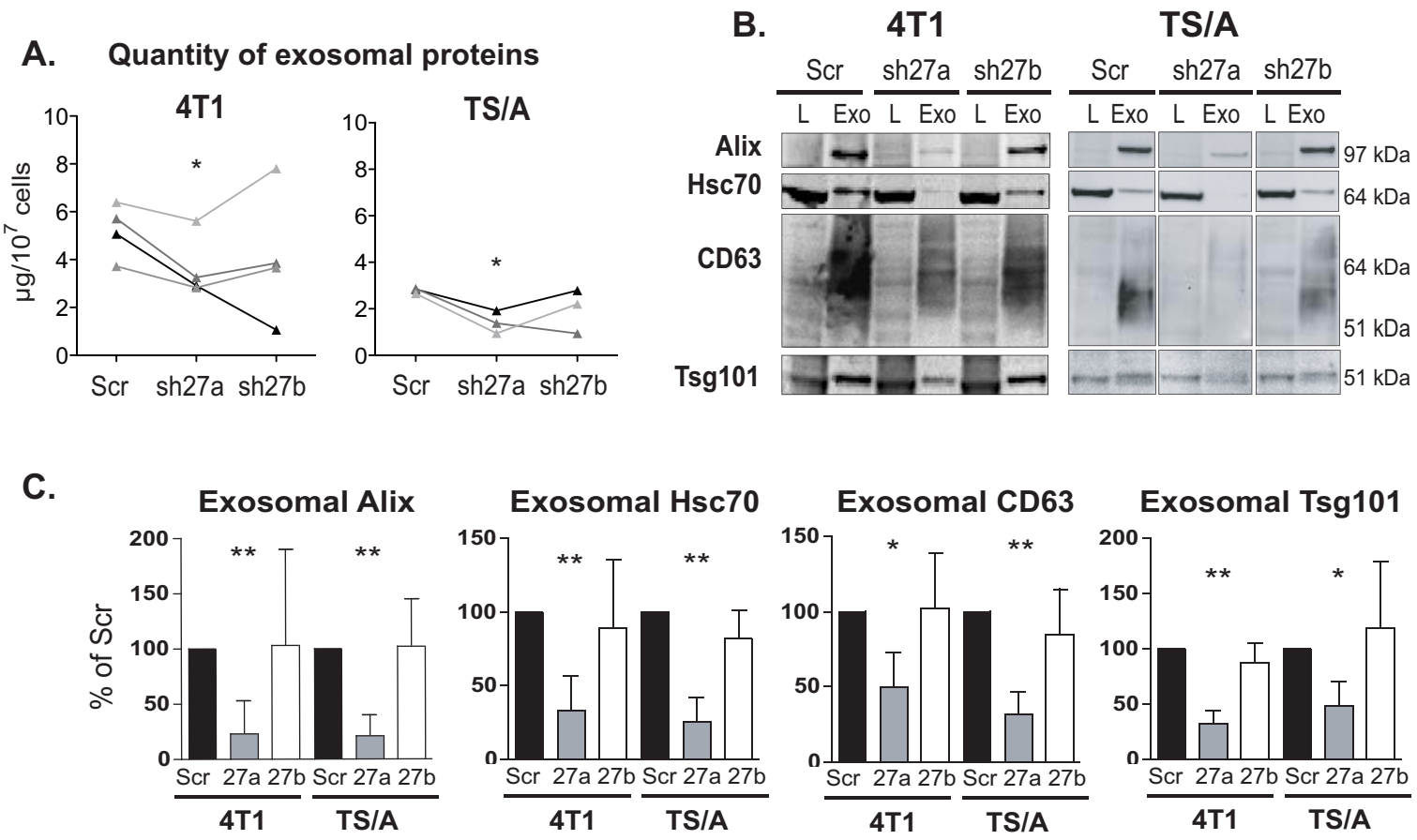


FIGURE 2



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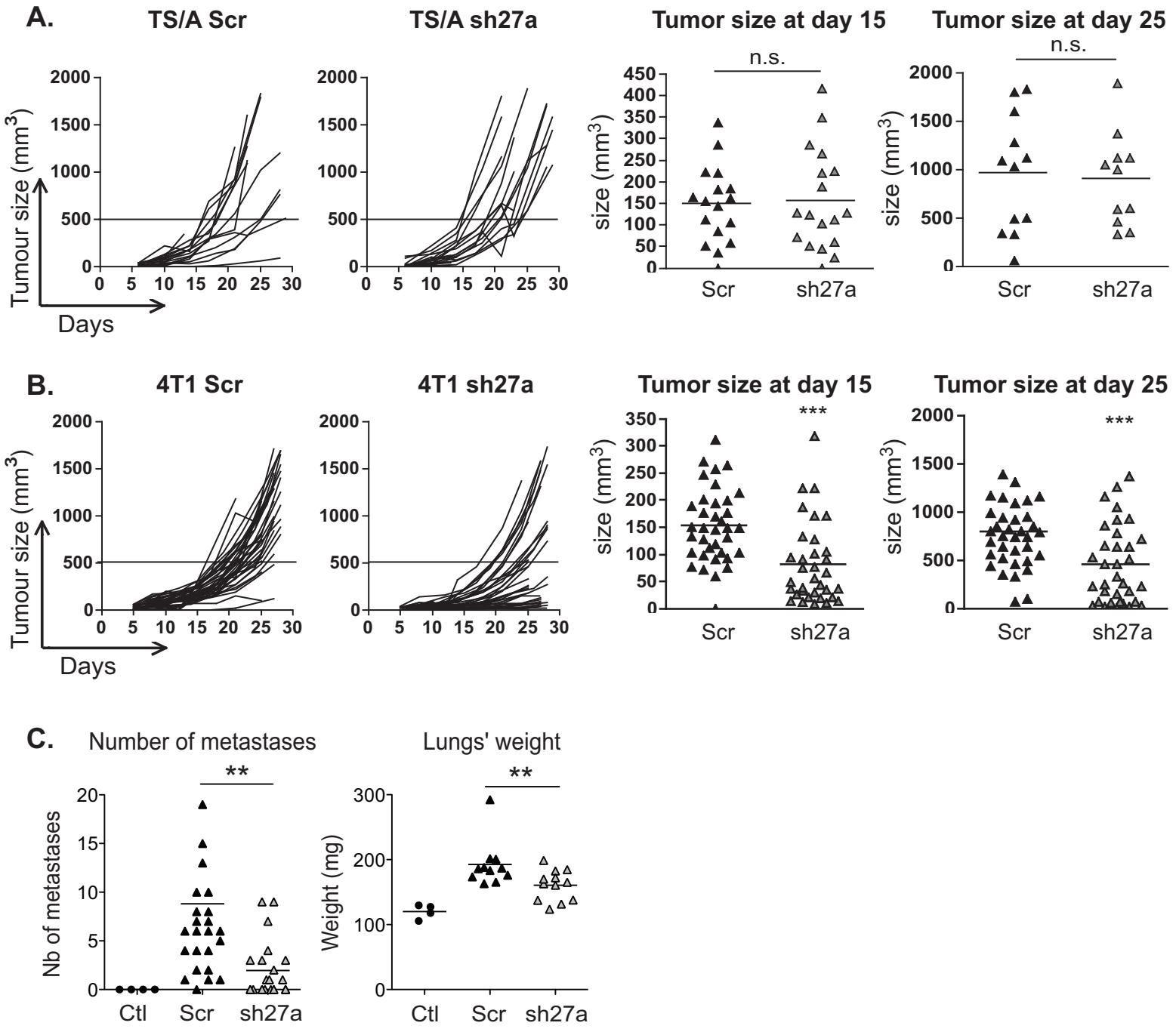

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FIGURE 4

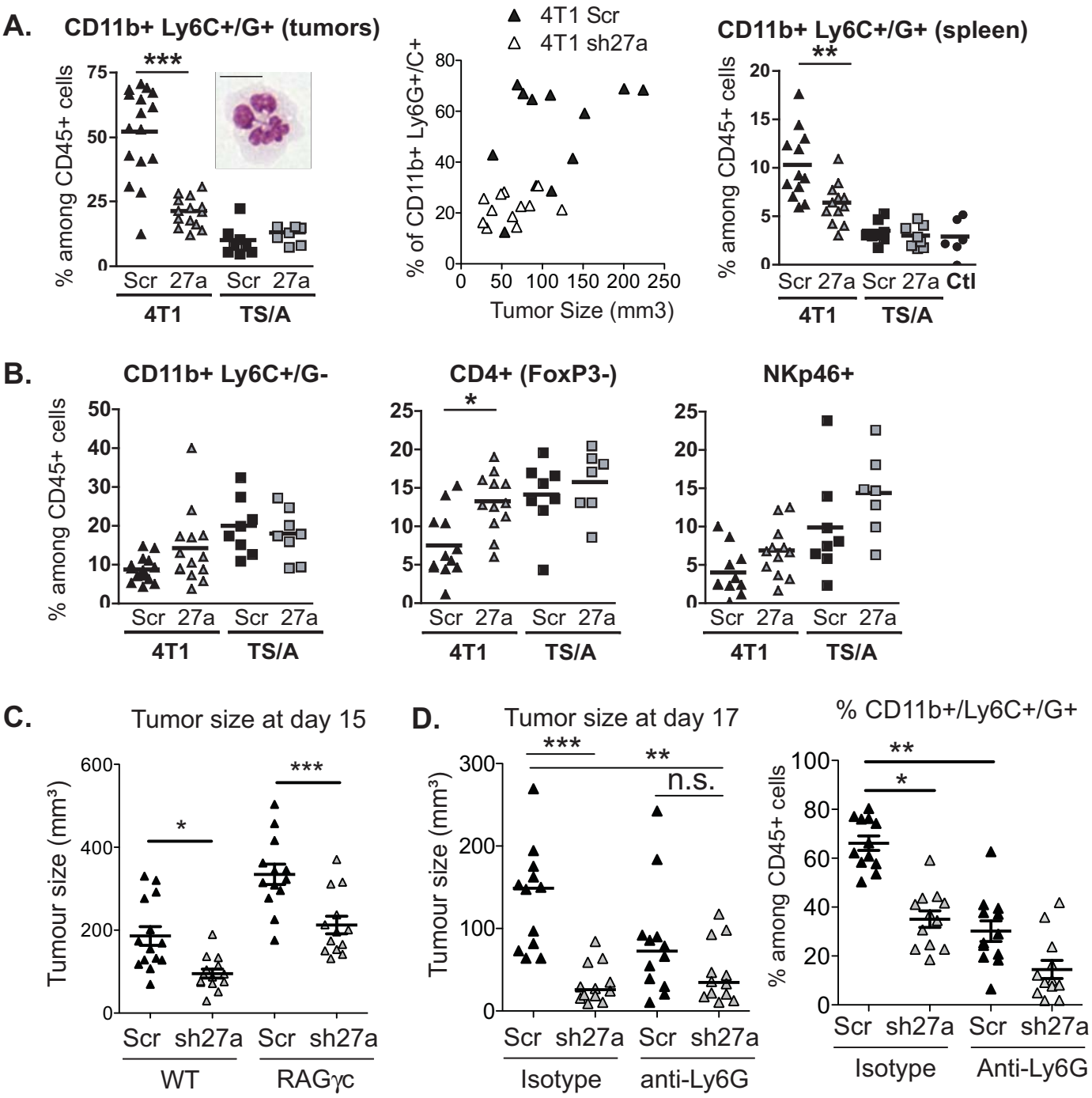

FIGURE 5

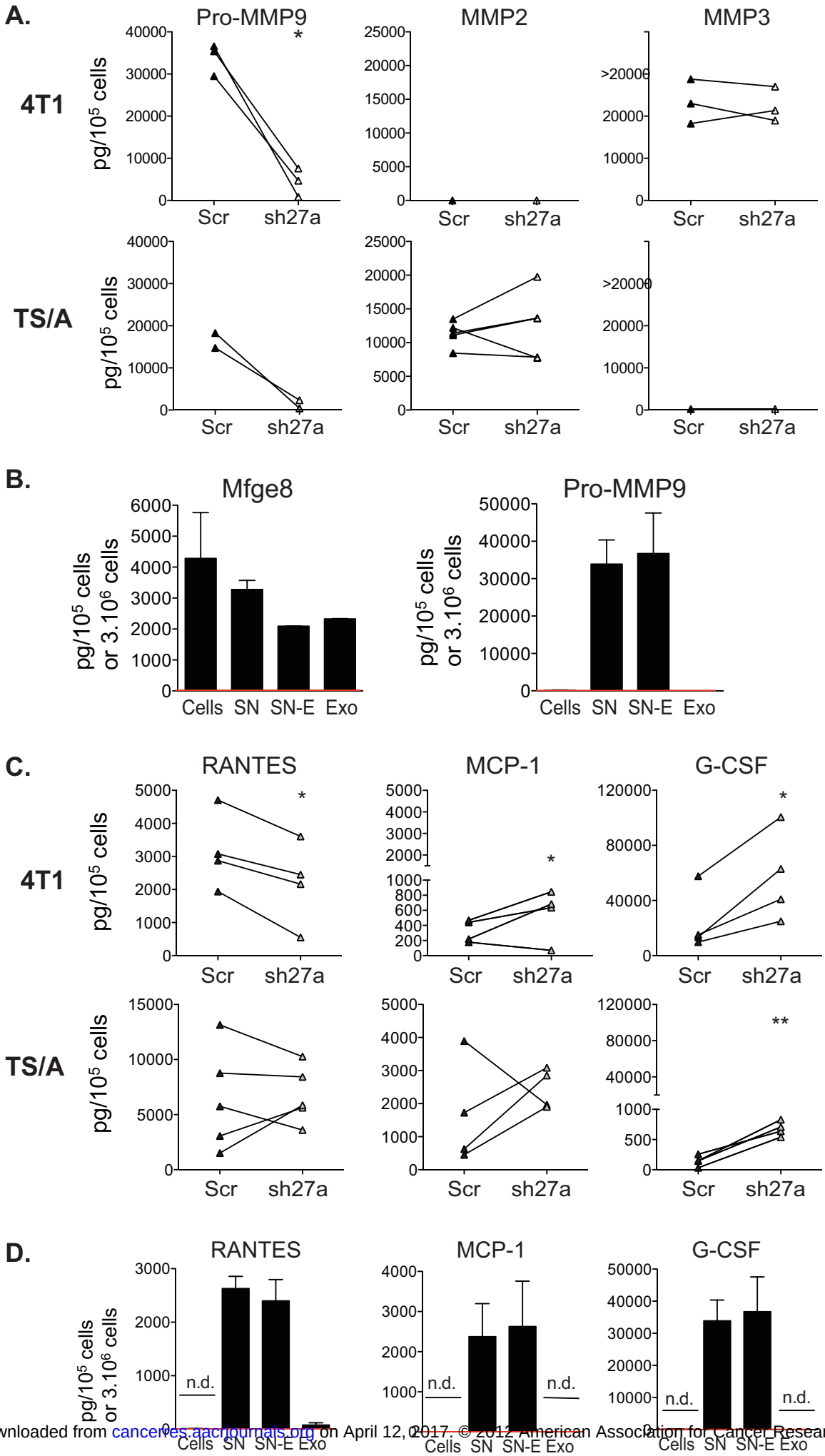

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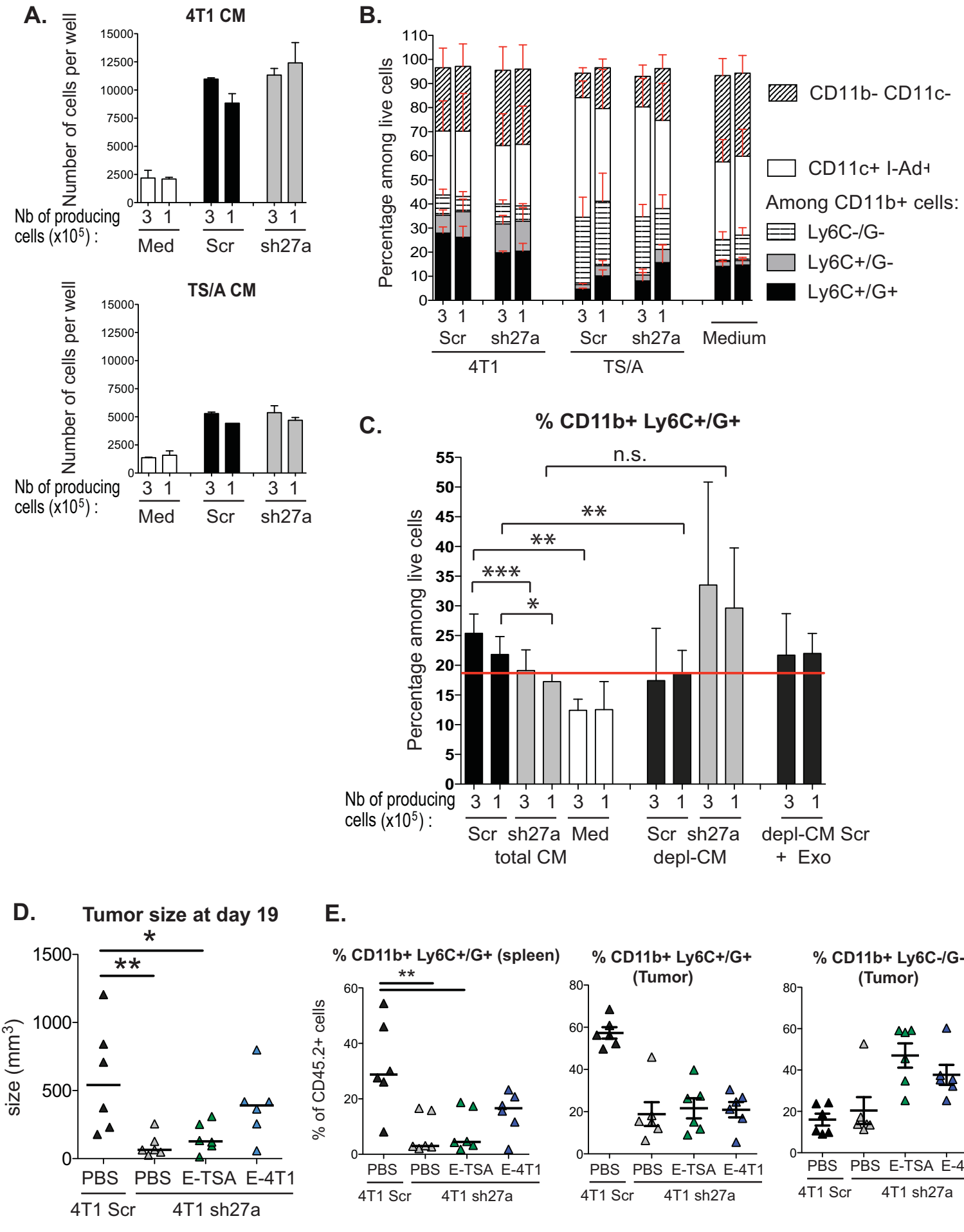
FIGURE 6



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