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Microenvironment and Immunology

Cancer Research

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 that 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 lead to exosome secretion, in human HeLa cells. Here, we used mouse models to show that Rab27a blockade in mammary carcinoma cells decreased secretion of exosomes characterized by endocytic markers, but also of matrix metalloproteinase 9, 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 nonmetastatic 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. *Cancer Res; 72(19); 4920–30. ©2012 AACR.*

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, that is, 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 (50–1,000 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

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exosomes from CD63-containing compartments was strongly decreased if expression of either RAB27A or RAB27B was knockeddown by short hairpin RNA (shRNA), whereas secretion of a soluble protein through the constitutive secretion pathway was not affected. We thus knockeddown Rab27a/b in mouse tumor cells to address the physiologic functions of exosome secretion *in vivo*.

Our results show that, in 2 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 protumoral 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 nonexosome-associated proteins by the 2 cell lines, especially the prometastatic matrix metalloproteinase 9 (MMP9). Finally, we show complementary effects of exosomes, soluble cytokines, and/or metalloproteinases 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 protumoral 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 wild-type controls were obtained from E. Vivier (CIML). 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 (American Type Culture Collection origin) and TS/A from L. Zitvogel (Institut Gustave Roussy). 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 1 month after lentivirus infection. Independent experiments were carried out with batches of independently infected cells.

Reagents

Detailed list of antibodies for fluorescence-activated cell sorting (FACS) and Western blotting is given as supplementary materials. Mouse Anti-Rab27a was generated in our laboratory (M. Seabra; ref. 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 ref. 16.

Exosome purification and characterization

Exosome purification was carried out 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 10–15 $\times$ 10^6 cells (<9 μg) or 30 μg of lysates (2–3 $\times$ 10^5 cells) were loaded on NuPAGE 4% to 12% BisTris gels (Invitrogen) and separated under nonreducing conditions (except for Rab27a analysis: reducing conditions, 150 μg of lysates).

Quantitative reverse transcriptase-PCR

Quantitative reverse transcriptase PCR (RT-PCR) was conducted 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 ($2^{Ct(GAPDH)-Ct(gene)} \times 1000$) 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 $\times$ width $\times$ [(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 alcohol/formalin/acetic acid and nodules were manually counted. Anti-Ly6G antibodies were injected everyday intraperitoneally 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 carried out with FlowJo software.

Analysis of secreted factors

Soluble secreted factors were analyzed in CM before exosome purification. CM depleted or not of exosomes (by 1 hour 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 (BD Biosciences), 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 nonculture-treated 24-well plates with tumor cell CM were cultured for 7 days with addition of fresh medium every 2 days. CM from 3 $\times$ 10^5 or 1 $\times$ 10^5 cells was added to 3.7 $\times$ 10^5 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)

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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 6 mouse tumor cells of different tissue origins. *Rab27a* mRNA was strongly expressed in the melanoma B16F10, whereas *Rab27b* was undetectable (Fig. 1A). In 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 2 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 (Fig. 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 nonmurine gene (Scr), to infect the 2 mammary carcinoma cell lines. Among 5 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 (Supplementary Fig. S1, Fig. 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 (Fig. 1D).

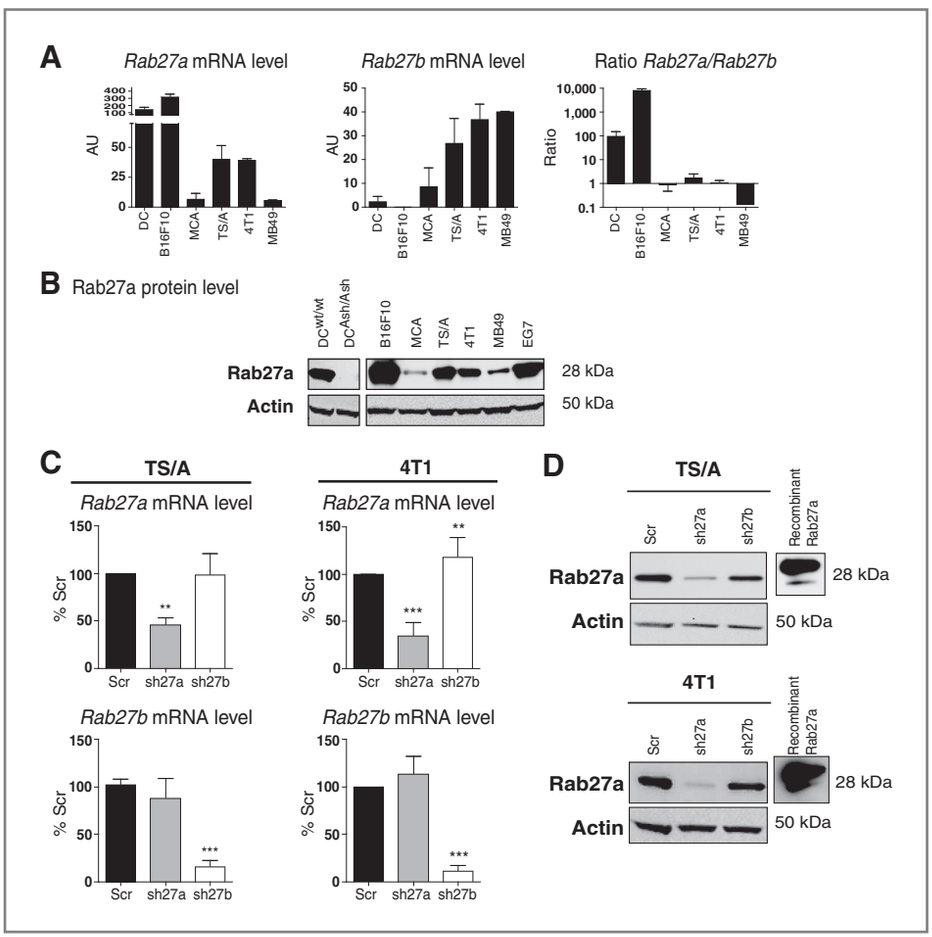

Figure 1. Rab27a and Rab27b are differently expressed in mouse tumor cell lines, and shRNA can inhibit their expression in mammary tumor cells. A, expression of *Rab27a* and *Rab27b* by quantitative RT-PCR in mouse dendritic cells (DC), and tumor cell lines: B16F10, MCA101 (MCA), TS/A, 4T1, MB49. mRNA level of *Rab27a* (left) or *Rab27b* (middle) is expressed in arbitrary units as compared with *Gapdh*, and the relative level of *Rab27a* and *Rab27b* is calculated by the ratio of arbitrary unit (right). Mean + SD from 1 (MB49), 2 (4T1, B16F10), 3 (DC), or 4 (MCA, TS/A) experiments. B, expression of Rab27a protein cell lysates from wild type (DC^{wt}) or Rab27a mutant (DC^{Ash}) 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 with control shRNA (Scr). Percentage of expression level in control cells, mean + SD 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 with control shRNA (Scr). Actin expression is shown as loading control. AU, arbitrary unit; DC, dendritic cells; MCA, methylcholanthrene.

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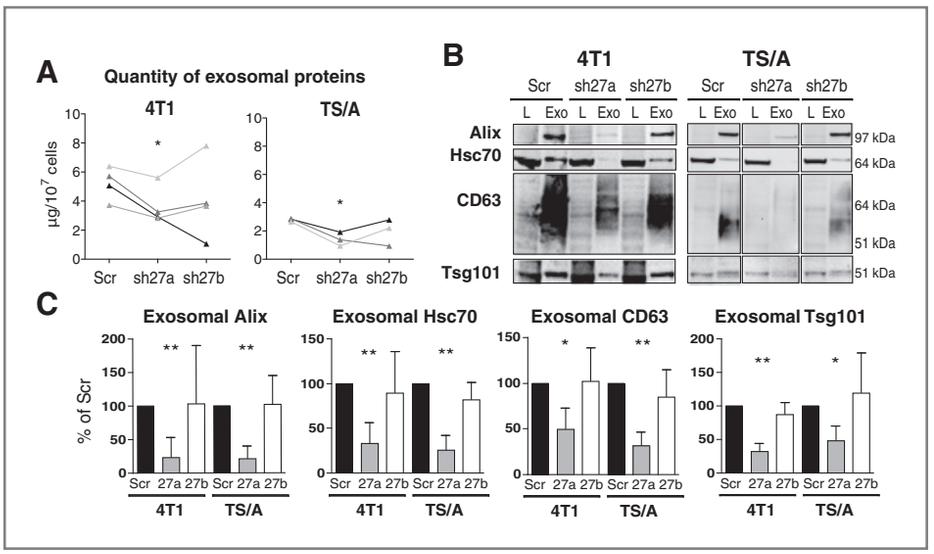

Figure 2. Inhibition of Rab27a, but not of Rab27b, decreases secretion of exosomes. A, total amount of exosomal proteins obtained from control (Scr), Rab27a-impaired (sh27a), or Rab27b-impaired (sh27b) 4T1 (left) or TS/A cells (right) quantified in 3 to 4 independent experiments. *, $P < 0.05$, paired Student *t* test. B and C, Western blot characterization of exosomes (Exo) secreted by control (Scr), sh27a- or sh27b-expressing 4T1 and TS/A. Cell lysates (L) were analyzed in parallel. One representative Western blot (B) and pooled quantifications (C) of exosomes obtained from sh27-expressing cells as compared with control cells are shown (mean + SD of 4–7 independent experiments). *, $P < 0.05$; **, $P < 0.01$; ANOVA with Dunnett's posttest.

Inhibition of Rab27a, but not Rab27b, affects exosome secretion

Exosomes were purified by differential ultracentrifugation (17) from conditioned culture medium (CM) 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 (Fig. 2A). We observed a consistent reduction of this amount in both TS/A and 4T1 cells expressing sh27a (Fig. 2A). In contrast, cells expressing

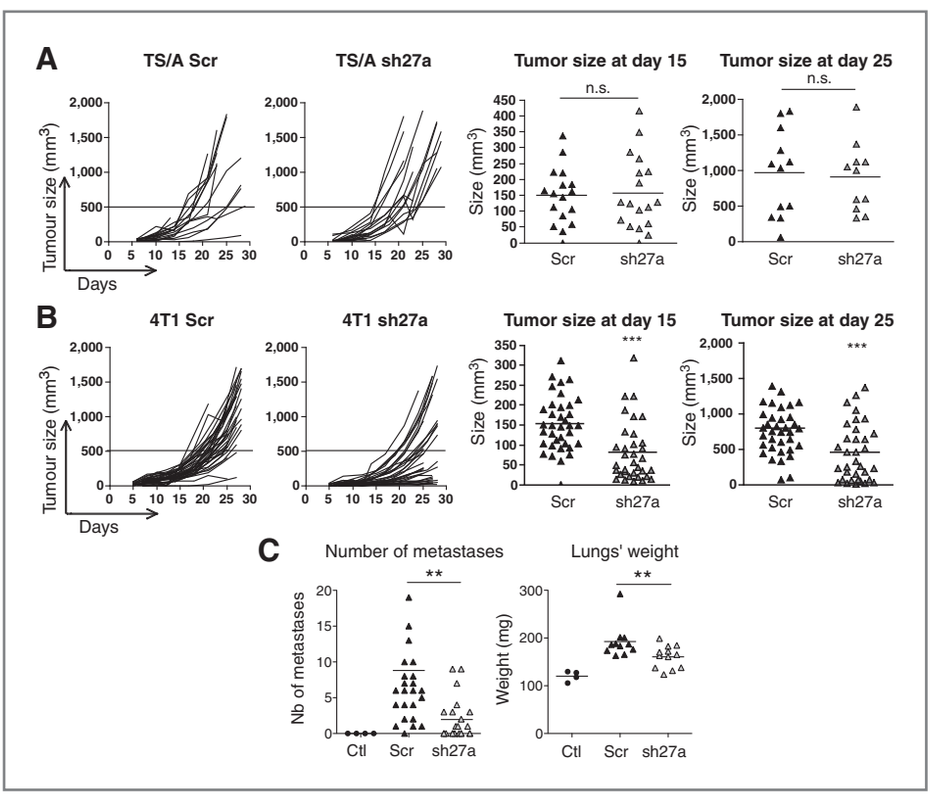

Figure 3. Local growth and metastases formation by 4T1 (but not by TS/A) is decreased by inhibition of Rab27a. A and B, control (Scr) or sh27a-expressing TS/A (A) or 4T1 (B) tumors were injected subcutaneously in the mammary area of syngeneic Balb/c mice. Tumor growth over 4 weeks (left) and tumor size at d15 and d25 (right) 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 *t* test). n.s., not significant. 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, nontumor injected mice. Results from individual mice pooled from 4 experiments are shown. *, $P < 0.05$; **, $P < 0.01$; One-way ANOVA, Dunnett's posttest.

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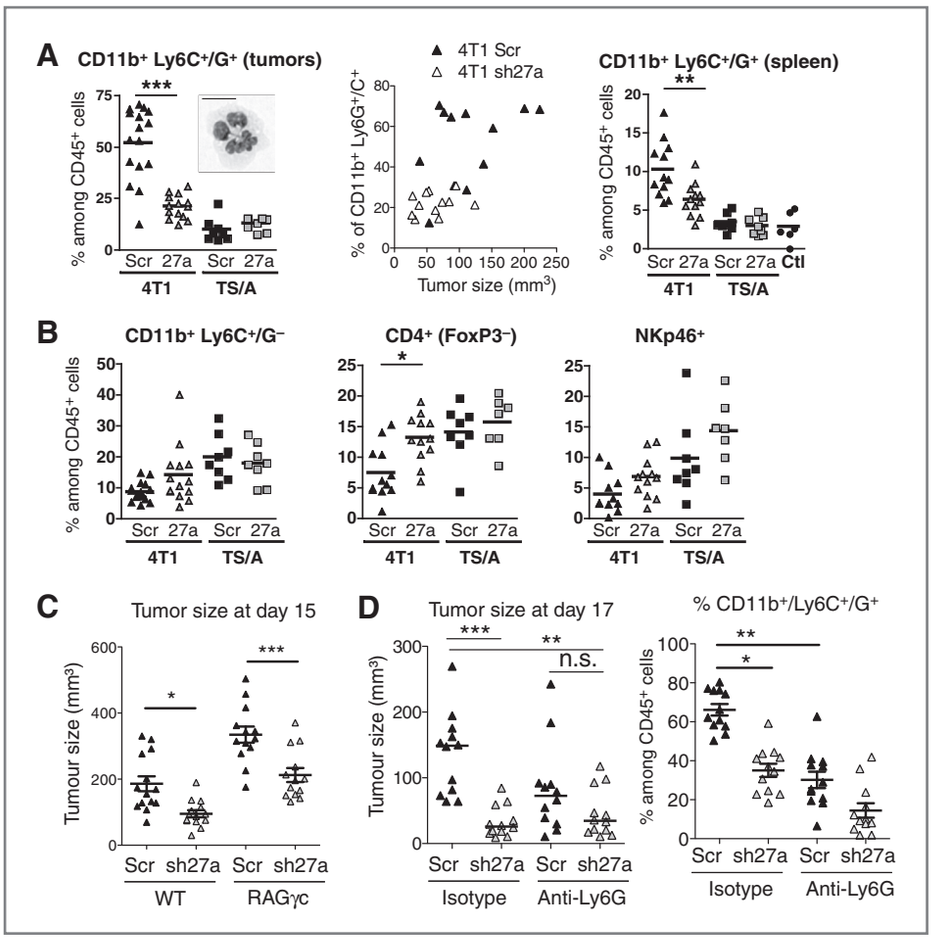

Figure 4. Rab27a inhibition prevents systemic accumulation of tumor-promoting neutrophils in mice bearing 4T1 tumors. A, quantification of $CD11b^+/Ly6C^+/G^+$ cells (inset, typical neutrophil aspect in cytospin; scale bar, 10 μm) in individual tumors (left) or spleen (right) of mice bearing Scr and sh27a-4T1 or TS/A at d13 to 15 after implantation (left), and as a function of tumor size in 4T1 (middle). 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 posttest. C, growth of control (Scr) and sh27a-expressing 4T1 tumors in $Rag2^{-/-}/\gamma c^{-/-}$ as compared with 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 with control antibody-injected mice (isotype). Tumor size at d15 (left) and mobilization of neutrophils in tumors (right) are represented. Growth of Scr-4T1 tumor becomes similar to growth of sh27a-4T1 upon neutrophil depletion (*, $P < 0.05$; ***, $P < 0.001$; Student t test). Results from individual mice pooled from 2 to 3 independent experiments are shown. n.s., not significant.

sh27b secreted variable amounts of exosomal proteins, not significantly different from what was released by control cells. Analysis of 4 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 (Fig. 2B and C). Thus, Rab27a is required for secretion of vesicles bearing endosomal markers (CD63, Alix, Tsg101), that is, corresponding to the original definition of exosomes. Rab27a therefore plays a similar role in the 2 mouse mammary carcinoma cells and in the human cervical carcinoma cell HeLa (13). Rab27b, in 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 nonendosome markers (Mfge8 and CD9) is not affected by Rab27a inhibition (18).

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

Because 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 (Fig. 3A). In contrast, Rab27a-impaired 4T1 tumors grew significantly more slowly than control (Scr) 4T1 cells (Fig. 3B), and induced lower number of lung metastases (Fig. 3C). Lower incidence of metastases was also observed after intravenous injection of the

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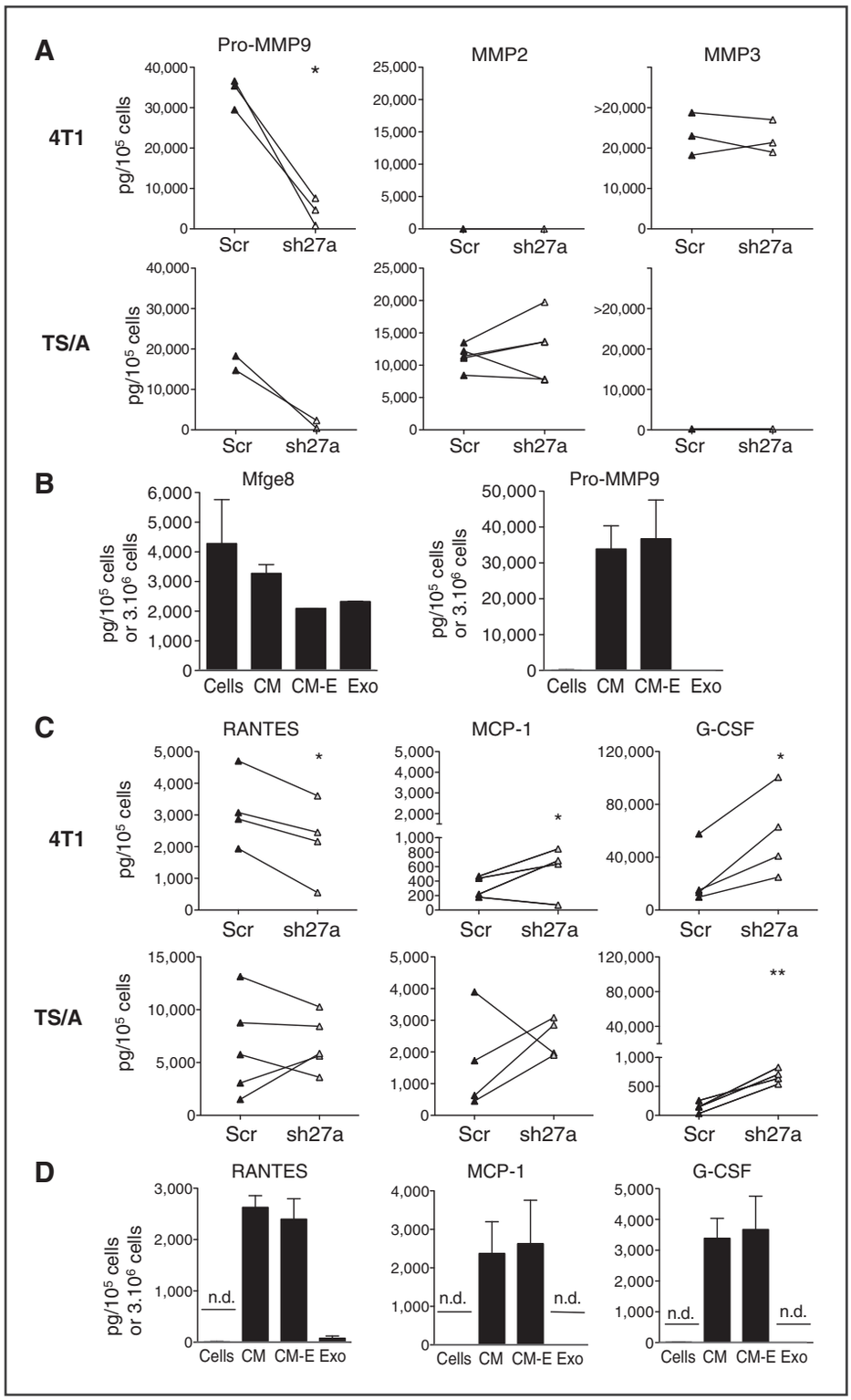

Figure 5. 4T1 and TS/A secrete different patterns of nonexosome bound proteins, some of which are modified upon invalidation of Rab27a. Quantification of proteins in CM from Scr or sh27a-expressing 4T1 (top) and TS/A (bottom). Individual results from 3 to 4 independent experiments are shown. *, $P < 0.05$; **, $P < 0.01$; Student *t* test. A, ELISA for pro-MMP9, MMP3, and MMP2. B, quantification of pro-MMP9 and Mfge8 in total cell lysates (Cells), CM, CM after 100,000 g ultracentrifugation (CM-E), and 100,000 g pellets (Exo) from 4T1 cells. C and 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.

sh27a-4T1 tumors (Supplementary Fig. S2A), showing that decreased metastatic ability of the locally growing sh27a-tumors was not simply because of smaller size of the subcutaneous tumors, but also due to reduced ability to colonize lungs. The difference in *in vivo* growth was not due to intrinsic slower cell proliferation, as sh27a- and Scr-4T1 cells grew with identical rates *in vitro* (Supplementary Fig. S2B).

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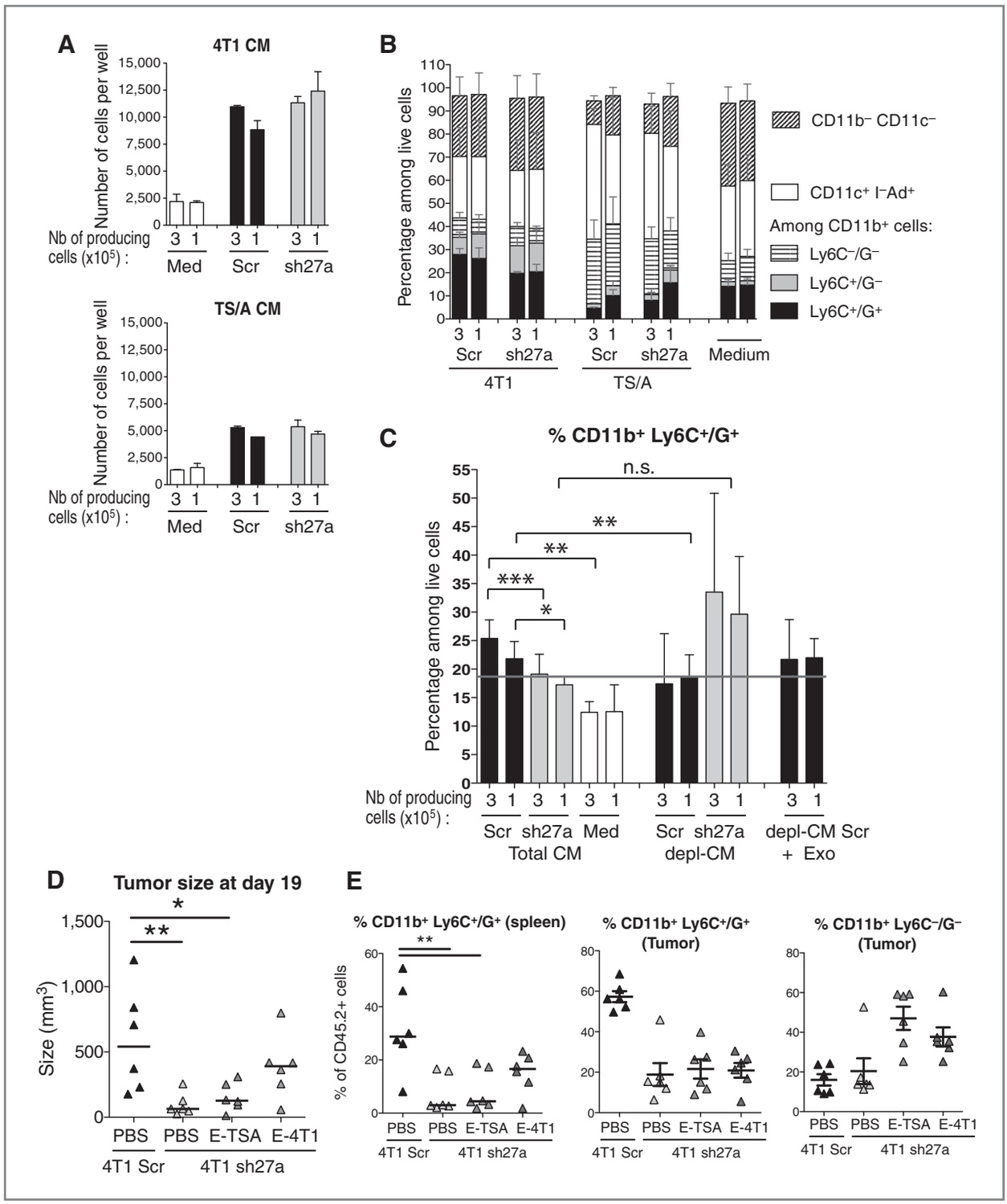

Figure 6. Soluble and particulate factors secreted by 4T1 affect *in vitro* survival and differenciation of bone marrow-derived neutrophils. A—C, bone marrow cells were cultured for 7 days in the presence of non-CM (Med) or CM from Scr- or sh27a-expressing 4T1 or TS/A (2 dilutions, corresponding to CM of 1–3.10^5 cells). A, number of live cells. Mean + SD in 1 representative experiment out of 3. B, nature of the cells (average% + SEM from 3 experiments): nonmyeloid 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 + SD of 2

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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 immunologic side. Analysis of immune cell populations in tumor-bearing mice showed strong infiltration of Scr-4T1 tumors by cells coexpressing the markers CD11b, Ly6C, and Ly6G (up to 70% of the immune cells, Fig. 4A, Supplementary Fig. S3A) displaying multilobed 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 (Fig. 4A middle panel). Other immune cells were concomitantly upregulated in sh27a-4T1 tumors, but the difference was statistically significant only for $CD4^+$ T lymphocytes (Fig. 4B). Neutrophils were also significantly more abundant in the spleen (Fig. 4A) and blood (not shown) of Scr-4T1 as compared with 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 natural killer (NK) lymphocytes (Fig. 4A and 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 Fig. S3B and S3C), of all adaptive and NK cells ($Rag2^{-/-}\gamma c^{-/-}$ hosts, Fig. 4C), or of neutrophils (depletion by anti-Ly6G antibody, Fig. 4D). Both Scr- and sh27a-4T1 grew more efficiently in $Rag2^{-/-}\gamma c^{-/-}$ than wild-type 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 (Fig. 4C, Supplementary Fig. S3B and S3C), thus showing that the protumoral effect of Rab27a does not involve these immune cells. In contrast, inhibition of neutrophil invasion strongly impaired growth of Scr-4T1 tumor, which grew similarly as neutrophil-poor sh27a-4T1 (Fig. 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 nonexosome 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 (Supplementary Fig. S4). Twenty-three proteins were secreted above the background level, with differences in the secretomes of 4T1 and TS/A, especially extracellular proteinases (pro-MMP9, MMP2, and MMP3), and cytokines and chemokines modulating myeloid cells and neutrophils (G-CSF/Csf3, MCP-1/Ccl2, RANTES/Ccl5; refs. 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 (Fig. 5A). Because 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 (Fig. 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 (Fig. 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 [granulocyte colony-stimulating factor (G-CSF)], and at lower levels the myeloid cell chemotactic factors MCP-1 and RANTES (Fig. 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. Such as for MMP9, none were secreted in association with vesicles (Fig. 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. Because 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* (Fig. 4). But impaired accumulation of neutrophils in sh27a-4T1 tumors cannot be explained by inhibition of G-CSF secretion, because this tumor secretes even more G-CSF than Scr-4T1.

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

experiments. Paired Student *t* test *, $P < 0.05$; **, $P < 0.01$; n.s., nonsignificant. D and E, growth (D, tumor size at d19) and percentage myeloid cells in spleens (E, left) and tumor (E, right) of Scr-4T1 or sh27a-4T1 after intratumoral injection of PBS or exosomes. Results from individual mice in 1 experiment are shown. **, $P < 0.01$; *, $P < 0.05$. One-way ANOVA with Bonferronni's posttest. 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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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 (Fig. 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 (Fig. 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 (Fig. 6B). The CM of TS/A, in contrast, promoted $CD11b^+/Ly6C^-/G^-$, and $CD11c^+/I\text{-}Ad^+$ 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 (Fig. 6C, CM), and more efficient at promoting $Ly6C^+/G^-$ cells (Fig. 6B). Complementation of the ultracentrifuged CM with its 100,000 g pellet (Fig. 6C, depl-CM+Exo) reconstituted the initial activity. Therefore, in a cytokine environment promoting survival of nondendritic 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 (Fig. 6D), and also increased systemic accumulation of neutrophils in spleen of tumor-bearing mice (Fig. 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 (Fig. 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 shows 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 2 murine tumor models, Rab27a is required for secretion of exosomes, but also of nonexosome-associated MMP9, whereas it inhibits secretion of a subset of cytokines. Similar observations have recently been published on the B16F10 melanoma cell line (12), where Rab27a shRNA decreased secretion of exosomes and also of some angiogenic growth factors (placenta growth factor, platelet-derived growth factor), while increasing secretion of protease inhibitors (tissue inhibitor of metalloproteinase 1). 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 conducting such analyses for proteinases (C. Recchi, M. Seabra and colleagues, 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 2 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 2 tumor cell lines of the same tissue origin. 4T1 (30) and TS/A (31) are 2 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 physiologic 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 granulocyte macrophage colony-stimulating factor *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 2 cell lines, but this led to decreased tumor growth *in vivo* of only one of them. On the basis of 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; ref. 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, Fig. 4A), but have also been used to characterize a population called myeloid-derived suppressor

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cells, which inhibit T lymphocyte activation (36). NK cells and the adaptive immune system, however, are not required for the Rab27a-dependent tumor promoting activity of 4T1 (Fig. 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 shown for efficient metastasis of 4T1 (38), thus the prometastatic effect of Rab27a in 4T1 is probably because of its role in MMP9 secretion. TS/A, in contrast, secretes another gelatinase, MMP2, whose secretion is not affected by Rab27a inhibition, and whose RAB27B-dependent role in degrading extracellular matrix and promoting invasion has been proposed in a human breast cancer cell line (39). Our observation that 2 different mouse mammary carcinoma secrete completely different patterns of metalloproteinases with different dependence on Rab27a thus suggests that the effect of Rab27a on tumor cell's ability to degrade the extracellular matrix, such as the effect on the immune system, will vary from one cell to another.

In conclusion, our work conclusively shows 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Bobrie, F. Reyal, C. Théry
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References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
2. Thery C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 2009;9:581–93.
3. Anderson HC, Mulhall D, Garimella R. Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis. Lab Invest 2010;90:1549–57.
4. Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Exosomes/microvesicles as a mechanism of cell-to-cell communication. Kidney Int 2010;78:838–48.
5. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654–9.
6. Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, de Milito A, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. J Biol Chem 2009;284:34211–22.
7. Bobrie A, Colombo M, Raposo G, Thery C. Exosome secretion: molecular mechanisms and roles in immune responses. Traffic 2011;12:1659–68.
8. Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat Med 2001;7:297–303.
9. Zeelenberg IS, Ostrowski M, Krumeich S, Bobrie A, Jancic C, Boissonnas A, et al. Targeting tumor antigens to secreted membrane vesicles *in vivo* induces efficient antitumor immune responses. Cancer Res 2008;68:1228–35.
10. Iero M, Valenti R, Huber V, Filipazzi P, Parmiani G, Fais S, et al. Tumour-released exosomes and their implications in cancer immunity. Cell Death Differ 2008;15:80–8.
11. Taylor DD, Gercel-Taylor C. Exosomes/microvesicles: mediators of cancer-associated immunosuppressive microenvironments. Semin Immunopathol 2011;33:441–54.
12. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med 2012;18:883–91.
13. Ostrowski M, Carmo NB, Krumeich S, Fanget I, Raposo G, Savina A, et al. Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol 2010;12:19–30.
14. Tolmachova T, Anders R, Stinchcombe J, Bossi G, Griffiths GM, Huxley C, et al. A general role for Rab27a in secretory cells. Mol Biol Cell 2004;15:332–44.
15. Fukuda M. Regulation of secretory vesicle traffic by Rab small GTPases. Cell Mol Life Sci 2008;65:2801–13.
16. Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepfer AM, Hinkle G, et al. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell 2006;124:1283–98.
17. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 2006;Chapter 3:Unit 3.22.

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18. Bobrie A, Colombo M, Krumeich S, Raposo G, Thery C. Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation. J Extracellular Vesicles 2012;1:18397.
19. Yu S, Liu C, Su K, Wang J, Liu Y, Zhang L, et al. Tumor exosomes inhibit differentiation of bone marrow dendritic cells. J Immunol 2007;178:6867–75.
20. Liu C, Yu S, Zinn K, Wang J, Zhang L, Jia Y, et al. Murine mammary carcinoma exosomes promote tumor growth by suppression of NK cell function. J Immunol 2006;176:1375–85.
21. Kowanetz M, Wu X, Lee J, Tan M, Hagenbeek T, Qu X, et al. Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C +granulocytes. Proc Natl Acad Sci U S A 2010;107:21248–55.
22. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 2011;475:222–5.
23. Taraboletti G, D'Ascenzo S, Borsotti P, Giavazzi R, Pavan A, Dolo V. Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. Am J Pathol 2002;160:673–80.
24. Vrijsen KR, Sluijter JP, Schuchardt MW, van Balkom BW, Noort WA, Chamuleau SA, et al. Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells. J Cell Mol Med 2010;14:1064–70.
25. Oshima K, Aoki N, Kato T, Kitajima K, Matsuda T. Secretion of a peripheral membrane protein, MFG-E8, as a complex with membrane vesicles. Eur J Biochem 2002;269:1209–18.
26. Desnos C, Schonn JS, Huet S, Tran VS, El-Amraoui A, Raposo G, et al. Rab27A and its effector MyRIP link secretory granules to F-actin and control their motion towards release sites. J Cell Biol 2003;163:559–70.
27. Yi Z, Yokota H, Torii S, Aoki T, Hosaka M, Zhao S, et al. The Rab27a/granuphilin complex regulates the exocytosis of insulin-containing dense-core granules. Mol Cell Biol 2002;22:1858–67.
28. Herrero-Turrion MJ, Calafat J, Janssen H, Fukuda M, Mollinedo F. Rab27a regulates exocytosis of tertiary and specific granules in human neutrophils. J Immunol 2008;181:3793–803.
29. Johnson JL, Brzezinska AA, Tolmachova T, Munafo DB, Ellis BA, Seabra MC, et al. Rab27a and Rab27b regulate neutrophil azurophilic granule exocytosis and NADPH oxidase activity by independent mechanisms. Traffic 2010;11:533–47.
30. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 1992;52:1399–405.
31. Nanni P, de Giovanni C, Lollini PL, Nicoletti G, Prodi G. TS/A: a new metastasizing cell line from a BALB/c spontaneous mammary adenocarcinoma. Clin Exp Metastasis 1983;1:373–80.
32. Rakhmilevich AL, Janssen K, Hao Z, Sondel PM, Yang NS. Interleukin-12 gene therapy of a weakly immunogenic mouse mammary carcinoma results in reduction of spontaneous lung metastases via a T-cell-independent mechanism. Cancer Gene Ther 2000;7:826–38.
33. Xiang X, Poliakov A, Liu C, Liu Y, Deng ZB, Wang J, et al. Induction of myeloid-derived suppressor cells by tumor exosomes. Int J Cancer 2009;124:2621–33.
34. Liu Y, Xiang X, Zhuang X, Zhang S, Liu C, Cheng Z, et al. Contribution of MyD88 to the tumor exosome-mediated induction of myeloid derived suppressor cells. Am J Pathol 2010;176:290–9.
35. Chalmin F, Ladoire S, Mignot G, Vincent J, Bruchard M, Remy-Martin JP, et al. Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. J Clin Invest 2010;120:457–71.
36. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009;9:162–74.
37. Yan HH, Pickup M, Pang Y, Gorska AE, Li Z, Chytil A, et al. Gr-1^{+}CD11b^{+} myeloid cells tip the balance of immune protection to tumor promotion in the premetastatic lung. Cancer Res 2010;70:6139–49.
38. Fayard B, Bianchi F, Dey J, Moreno E, Djaffer S, Hynes NE, et al. The serine protease inhibitor protease nexin-1 controls mammary cancer metastasis through LRP-1-mediated MMP-9 expression. Cancer Res 2009;69:5690–8.
39. Hendrix A, Maynard D, Pauwels P, Braems G, Denys H, Van den Broecke R, et al. Effect of the secretory small GTPase Rab27B on breast cancer growth, invasion, and metastasis. J Natl Cancer Inst 2010;102:866–80.

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Rab27a Supports Exosome-Dependent and -Independent Mechanisms That Modify the Tumor Microenvironment and Can Promote Tumor Progression

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