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Expression of αvβ3- or αvβ5-integrins and selectins is widespread on blood cells and endothelial cells. Here we report that human tumor cells injected s.c. into mice lacking β3- or β3/β5-integrins or various selectins show enhanced tumor growth compared with growth in control mice. There was increased angiogenesis in mice lacking β3-integrins, but no difference in structure of the vessels was observed by histology or by staining for NG2 and smooth muscle actin in pericytes. Bone marrow transplants suggest that the absence of β3-integrins on bone marrow-derived host cells contributes to the enhanced tumor growth in β3-null mice, although few, if any, bone marrow-derived endothelial cells were found in the tumor vasculature. Tumor growth also was affected by bone marrow-derived cells in mice lacking any one or all three selectins, implicating both leukocyte and endothelial selectins in tumor suppression. Reduced infiltration of macrophages was observed in tumors grown in mice lacking either β3-integrins or selectins. These results implicate cells of the innate immune system, macrophages or perhaps natural killer cells, in each case dependent on integrins and selectins, in tumor suppression.

tumors | bone marrow transplants | cell adhesion | innate immunity

We have previously investigated the growth and metastasis of transplanted tumors in mice lacking specific cell-adhesion receptors, namely integrins (1–3) or selectins (4–6).

In mice lacking αv-integrins (αvβ3 and αvβ5), tumors grow larger; this growth is accompanied by enhanced tumor angiogenesis (2). However, it is unclear whether other factors, such as altered vessel morphology, pericyte recruitment, or altered immune responses in the integrin-deficient mice, may contribute to the enhanced tumor growth.

In the case of selectins, we have shown that experimental metastases to the lungs are reduced in mice lacking P- and/or L-selectins (4–6). This reduction is believed to result from the tumor cells' expression of ligands for selectins such that selectins on host platelets, leukocytes, or endothelial cells can bind these ligands and promote metastatic arrest in the lungs after tail vein injection (7–10). Examination of this hypothesis using a more complete model of metastasis, such as metastasis from a s.c. site, would be desirable.

Given these two prior lines of investigation and the questions they raised, we have investigated further the s.c. growth of xenotransplanted tumors in mice lacking various combinations of integrins or selectins. We report here results indicating that both β3-integrins and selectins contribute to host responses suppressing tumor growth. In each case, bone marrow (BM)-derived host cells, dependent on integrins or selectins, seem to inhibit tumor growth, and in the absence of these adhesion receptors tumors grow significantly larger.

Materials and Methods

Mice. All mice were generated in our own laboratory. *β3-Integrin*-null mice (2, 11–12) were intercrossed with mice lacking the *Rag2* gene (13) and with mice lacking β5-integrin (14) to generate mice deficient in *Rag2*, in *Rag2* and *β3-integrin*, or in *Rag2*, *β3-*, and *β5-integrin* genes. Mice lacking all combinations of the three selectin genes (P-, L-, and E-selectins; ref. 15) were crossed with the *Rag2*-null mice to generate mice deficient in *Rag2* and various combinations of selectins. All mice used in the experiments described here were, therefore, *Rag2*-null on a mixed C57BL/6 $\times$ 129S4 background and lacked B, T, and NKT cells. Because of the complexities of the breeding, experimental mice having or lacking individual adhesion genes were cousins, not siblings. However, the effects seen were consistent among diverse experiments and highly significant. Mice were maintained under severe combined immunodeficient conditions in the Massachusetts Institute of Technology animal facility. All mice were viable and fertile and generally remained healthy for the duration of the experiments, despite occasional hemorrhage in β3-integrin-deficient mice (11) and late-onset dermatitis in mice lacking both endothelial selectins P and E (15, 16). In an earlier study of metastasis, tumor growth was reduced in P-selectin-deficient mice (4), but those mice were not kept in specific pathogen-free conditions and showed opportunistic infections and significant morbidity that apparently affected tumor growth.

Antibodies. Monoclonal antibodies used for immunohistochemistry were rat anti-platelet endothelial cell-adhesion molecule (PECAM)-1/CD31, rat anti-Pan-NK, biotinylated hamster anti-β3-integrin (all from PharMingen), mouse anti-smooth muscle α actin (Sigma), rat anti-F4/80 (Serotec), and rabbit anti-NG2 polyclonal antiserum (Chemicon); all were used at a 1:100 dilution. Secondary antibodies for immunofluorescence were Alexa Fluor-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, or goat anti-rat IgG (all from Molecular Probes). Biotin-conjugated goat anti-rat Ig antibody used for chromogenic analysis was from PharMingen. Fluorescence-activated cell sorter analyses used FITC-conjugated rat monoclonal antibodies anti-Pan-NK or anti-F4/80.

Immunohistochemistry and Histology. Five-micrometer frozen sections of tumors were used for immunohistochemistry. Chromogenic visualization followed the VECTASTAIN ABC kit protocol (Vector Laboratories). For immunofluorescence, the conjugated secondary antibodies were used at a 1:400 dilution. Histology was performed on 5-μm paraffin-embedded or 1- to 2-μm plastic-embedded tumor sections counterstained with hematoxylin/eosin or with methyl green, respectively. All images were visualized with a Zeiss Axiophot photoscope. Images were

Abbreviations: BM, bone marrow; PECAM-1, platelet endothelial cell-adhesion molecule 1.

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Flow Cytometry. FITC-conjugated rat monoclonal antibodies (anti-mouse Pan-NK or anti-F4/80) were used to stain natural killer (NK) cells or monocytes/macrophages present in the blood of mice. Blood samples were incubated with a 1:100 dilution of antibody for 1 h at room temperature, treated with red cell lysis buffer (17), washed twice with PBS, resuspended in PBS, and analyzed in a FACScan with CELLQUEST software (Becton Dickinson).

Mouse Tumor Transplants. Eight- to 12-week-old mice were injected s.c. with either 2×10^6 LS 180 human colon cancer cells (ATCC no. CL-187) or 2×10^6 A375 SM human melanoma cells (18). Tumors were harvested, weighed, and processed from 3 days to 5 weeks later. Every experiment was repeated at least 3 times.

Mouse BM Transplants. Eight-week-old mice (recipients) were lethally irradiated with 1,000 rads (a dose of 600 rads followed 4 h later by a dose of 400 rads; 1 rad = 0.01 Gy) and reconstituted with an intraorbital injection of 2×10^6 BM cells from donors of the desired genetic makeup. These mice were kept in sterile conditions and fed antibiotics for 10 days. Five weeks after the BM transplant, mice were injected s.c. with human tumor cells and analyzed as described above.

Statistical Analysis. Data were analyzed by using a program available at www.physics.csbsju.edu/stats. Tumor sizes are represented by box-and-whisker plots (Figs. 1, 3, 5, and 6) wherein each box represents an interquartile range (IQR; the central 50% of the data points), the horizontal line in each box represents the median, and vertical bars represent a spread of 1.5 × IQR. Dots represent outliers, which were included in calculations of significance; n indicates the number of samples, and p indicates the probability of identity of the distributions. In Fig. 5*a*, data from several experiments were combined and expressed as percentages of the mean WT value, and the box-and-whisker plots were generated by using EXCEL (Microsoft) (19).

Results

Tumor Growth Is Enhanced in β3- or β3/β5-Null Mice. Immunocompromised *Rag2*-null (control), *Rag2*/β3-null, and *Rag2*/β3/β5-null mice were injected s.c. with human tumor cells, either colon carcinoma (LS 180) or melanoma (A375 SM). As shown in Fig. 1, tumors grew in all three lines of mice, but tumor size was enhanced significantly in *Rag2*/β3- or *Rag2*/β3/β5-integrin-deficient mice when compared with *Rag2*-null mice WT for integrins ($P < 0.0001$) Differences in growth rates were seen as early as 2 weeks after implantation.

Angiogenesis Is Not Blocked in the Absence of β3- or β3/β5-Integrins. Sections of human tumors were analyzed at different times after the s.c. injections. No morphological differences were observed among the vessels within the different tumors. Immunohistochemical analysis with anti-PECAM-1, an endothelial cell marker, revealed an increased blood vessel density in the absence of β3-integrins (Fig. 2) or β3/β5-integrins (data not shown), as previously described for other tumors (2, 18). To analyze possible differences in the recruitment of vascular mural cells (pericytes and smooth muscle cells), sections of tumors were stained with antibodies against NG2 (Fig. 2) or smooth muscle α actin (data not shown), markers for pericytes, and costained with anti-PECAM-1 antibody to reveal the endothelial cells (Fig. 2 *Left*). Similar staining for NG2 or smooth muscle α actin was observed around the vessels of tumors grown in control or in β3- or β3/β5-null mice. Thus, the absence of these integrins does not block vessel development, which is in fact enhanced (≈17%) compared with that in the WT.

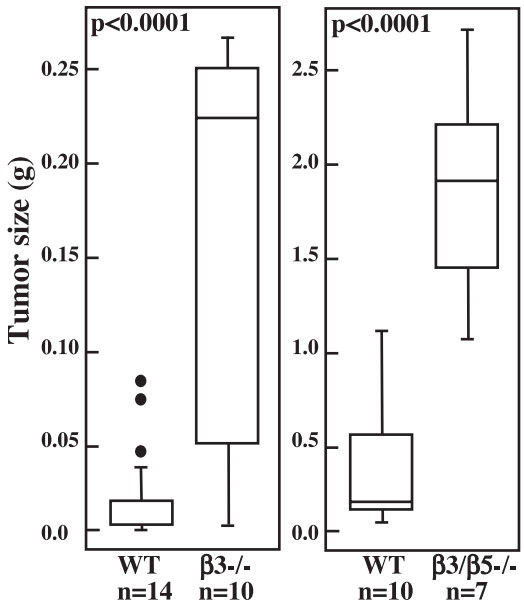

Fig. 1. Tumor growth is increased in β3- and β3/β5-integrin-deficient mice. Human A375 SM (*Left*) and LS 180 (*Right*) tumors grown s.c. in *Rag2*-null/$\beta3^{+/+}$ and *Rag2*/β3-null or *Rag2*/β3/β5-null mice. Box plots show tumor weights in grams for 4-week-old tumors (*Left*) and 3-week-old tumors (*Right*). $P < 0.0001$; n, the number of animals in each group.

Increased Tumor Growth in β3-Null Mice Is BM-Dependent. To determine whether BM cells were responsible for the different tumor growth in the various mouse lines, recipients (*Rag2*-null or *Rag2*/β3-null) were lethally irradiated and reconstituted with BM derived from mice with different genetic backgrounds. Five weeks after the BM transplants, these mice were injected s.c.

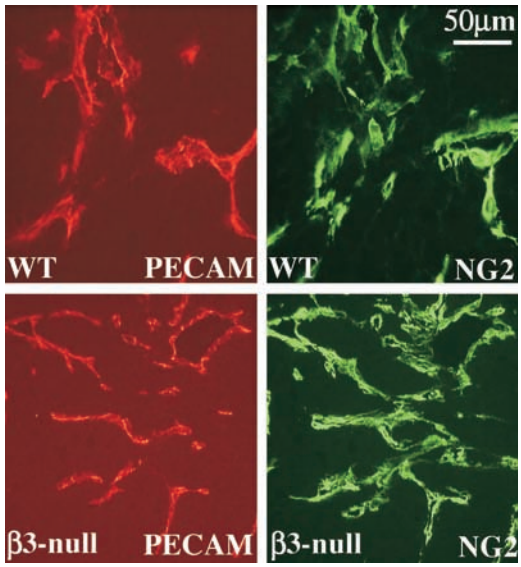

Fig. 2. Characterization of vasculature in human A375 SM and LS 180 tumors grown in *Rag2*-null/$\beta3^{+/+}$ or *Rag2*/β3-null mice. PECAM-1 and NG2 double-staining of 11-day-old LS 180 tumors from *Rag2*-null/$\beta3^{+/+}$ or *Rag2*/β3-null mice. PECAM-1 (red) and NG2 (green) staining are present around the same vessels. Similar results were obtained with smooth muscle α actin staining for pericytes and with tumors grown in *Rag2*/β3/β5-null mice (data not shown).

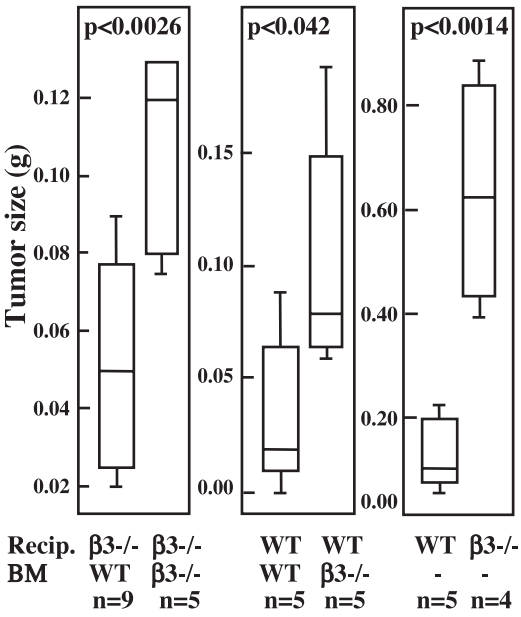

Fig. 3. Analysis of s.c. tumors in mice after BM transplantation. *Rag2*-null/$\beta3^{+/+}$ or *Rag2*/β3-null recipient (Recip.) mice were lethally irradiated and reconstituted with BM derived from *Rag2*/$\beta3^{+/+}$ or *Rag2*/β3-null mice and were injected s.c. 5 weeks later with LS 180 human tumor cells. The box plots represent the size of 3-week-old tumors. Reduced tumor growth can be observed in mice that received *Rag2*-null/$\beta3^{+/+}$ BM compared with mice that received *Rag2*/β3-null BM. (*Right*) Nonirradiated mice that did not receive a BM transplant are shown for comparison. (*Left*) $P < 0.0026$. (*Center*) $P < 0.042$. (*Right*) $P < 0.0014$. n, Number of animals in each group.

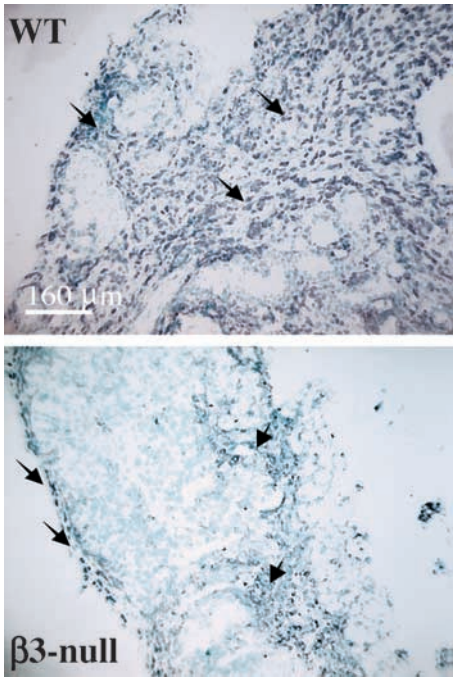

Fig. 4. Macrophage infiltration in A375 SM tumors grown in *Rag2*/$\beta3^{+/+}$ or *Rag2*/β3-null mice. Sections of 3-day-old A375 SM tumors were stained with an anti-macrophage antibody. A higher infiltration of macrophages (brown, indicated by arrows) was observed in tumors grown in control mice (*Upper*) compared with tumors grown in *Rag2*/β3-null mice (*Lower*). Note also the greater infiltration into the tumor in $\beta3^{+/+}$ mice. (Scale bar, 160 μm.)

with tumor cells. As shown in Fig. 3, tumor growth was enhanced only in mice reconstituted with BM cells derived from β3-null mice. Tumor growth was reduced in all irradiated compared with nonirradiated mice, independent of genetic makeup (note different scales). However, in all comparisons, mice with BM WT for integrins supported significantly less tumor growth than did mice with BM lacking β3-integrins. Therefore, BM-derived, integrin-dependent, host cell type(s) suppress tumor growth.

To investigate whether the increased tumor growth in animals that received BM transplants could be affected by endothelial cell precursors contributing to the excess vessel growth, we stained for β3-integrins. Tumors grown in β3-null animals showed no staining for β3-integrin on the vessels, whereas positive endothelial cell staining was observed on vessels of tumors grown in *Rag2*-null/$\beta3^{+/+}$ animals or in the same animals transplanted with $\beta3^{-/-}$ BM cells. However, when β3-null animals were transplanted with *Rag2*-null/$\beta3^{+/+}$ BM cells, no positive staining for β3-integrins was observed on the endothelial cells within the tumors (Fig. 8, which is published as supporting information on the PNAS web site). This suggests that all or most endothelial cells of the tumor vessels originate from local host vessels and not from potential BM-derived endothelial cell precursors. Thus, the effects of the transplants apparently reflect circulating blood cells rather than recruitment of endothelial progenitors.

Reduced β3-Null Macrophage Infiltration in Human Tumors. Because the *Rag2*-null mice used in these experiments do not develop mature B and T lymphocytes, increased tumor growth in animals lacking β3- or $\beta3/\beta5$-integrins could not be attributed to an alteration in the lymphocyte population. However, it is possible that other immune cells expressing β3- or β5-integrins were not functioning properly and therefore could not block tumor growth. We tested some cells involved in the innate immune response, namely macrophages and NK cells. We analyzed the recruitment of macrophages around and within the tumors by staining the human tumor sections with an anti-F4/80 antibody (macrophage antigen) at different times. As shown in Fig. 4, macrophage infiltration can be observed in tumors grown in mice WT for integrins 3 days after injection, and fewer macrophages are present around and within the tumors grown in β3- or $\beta3/\beta5$-null mice. Similar results were obtained at later times (data not shown). Because of the uneven distributions of macrophages, precise quantification was difficult, but microscopic counts suggested a 6- to 10-fold reduction in the integrin-deficient mice.

Immunohistochemistry experiments also were performed for NK cells by using an anti-Pan-NK antibody. The numbers of NK cells associated with the tumors were low in all animals, and we could not see obvious differences (data not shown). To test whether reduced recruitment was due to a reduced population of monocyte/macrophage lineage or NK cells in the blood of the β3-null animals, we performed fluorescence-activated cell sorter analyses. No decrease in the F4/80-positive population or in NK cells was observed in *Rag2*/β3-null compared with *Rag2*-null/$\beta3^{+/+}$ mice (data not shown).

Tumor Growth also Is Enhanced in Selectin-Deficient Mice. Immunocompromised *Rag2*-null (control) or *Rag2*-null/P-, E-, L-, or ELP-null mice were injected s.c. with the same two human tumor cell lines. As shown in Fig. 5*a*, tumors grew in all lines of mice, but tumor size was greatly enhanced in the absence of one or all three selectins when compared with that in controls. The most significant increase was observed in the triple-selectin-knockout mice. Again, differences in growth rates were seen as early as 2 weeks after implantation and accelerated greatly after 3 weeks. Interestingly, A375 SM tumors also showed enhanced growth in

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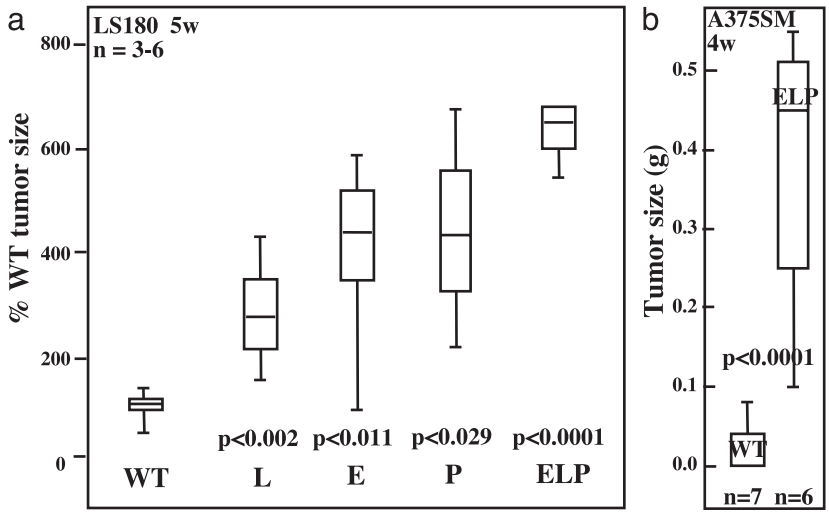

Fig. 5. Tumor growth is increased in selectin-deficient mice. (*a*) Human LS 180 s.c. tumors grown in *Rag2*-null or *Rag2*/L-, E-, P-, or ELP-null mice. (*b*) Human A375 SM s.c. tumors grown in WT or ELP-null mice. Box plots show the tumor weights as percentage of their size in selectin WT mice at 5 weeks (*a*) and in grams at 4 weeks (*b*).

triple-selectin-deficient mice (Fig. 5*b*), although these cells, unlike LS 180 cells, do not express selectin ligands (5).

Increased Tumor Growth in ELP-Null Mice Is Partially BM-Dependent. To determine whether BM-derived cells were responsible for the increased tumor growth in the ELP-null mice, we performed BM transplants as described above. Recipient *Rag2*-null or *Rag2*/ELP-null mice were lethally irradiated and reconstituted with BM derived from either *Rag2*-null or *Rag2*/ELP-null mice and then challenged with tumor cells. As before, tumor growth was reduced in all irradiated compared with nonirradiated mice (Fig. 6 *Left*; note the different scales). Tumor growth was enhanced only in mice reconstituted with BM cells derived from ELP-null mice (Fig. 6 *Center* and *Right*), suggesting the involvement of selectin-dependent, BM-derived cells in suppressing tumor growth. However, tumor growth in ELP-null mice that received a WT selectin BM transplant was slightly more pronounced than the growth in WT mice that received a WT BM transplant (compare Fig. 6 *Center* and *Right*; $P = 0.041$). This difference suggests that a BM-independent but selectin-dependent event also can affect tumor growth. Therefore, tumor growth is clearly affected not only by selectin-dependent, BM-derived cells, but also by selectins on BM-independent cells, probably endothelial cells; these express P- and E-selectins and are the only cells that express E-selectin, which clearly does have an effect (Fig. 5; see also *Discussion*).

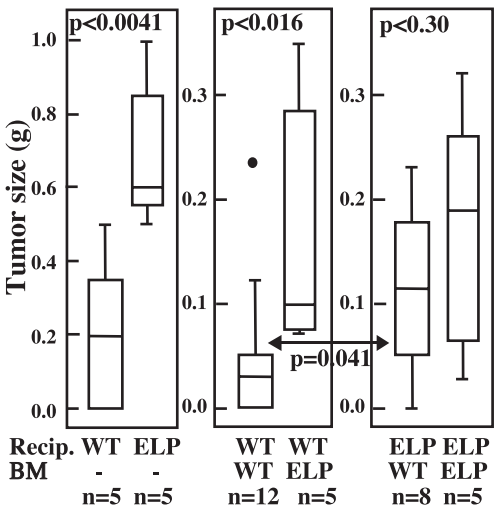

Fig. 6. Tumor growth is partially mediated by BM-derived cells in selectin-deficient mice. Human LS 180 s.c. tumors grown in mice that received BM transplants. (*Center* and *Right*) Control or selectin-null recipient (Recip.) mice were lethally irradiated and reconstituted with BM derived from either control or selectin-deficient mice and were injected s.c. 5 weeks later with LS 180 human tumor cells. (*Left*) Control mice were not irradiated and did not receive BM transplants. Box plots show the tumor weights in grams for 3-week-old tumors.

Reduced Macrophage Infiltration in ELP-Null Mice. We analyzed the infiltration of macrophages and NK cells by staining with anti-F4/80 (macrophage antigen) or anti Pan-NK antibody at different times. As shown in Fig. 7, macrophage infiltration can be observed around and within the tumors grown in *Rag2*-null

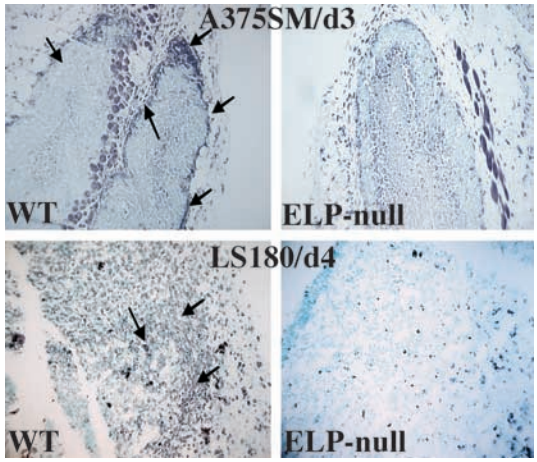

Fig. 7. Macrophage infiltration in tumors grown in *Rag2*-null or *Rag2*/ELP-null mice. Sections of 3-day-old A375 SM (*Upper*) and 4-day-old LS 180 (*Lower*) tumors were stained with an anti-macrophage antibody. A higher infiltration of macrophages (purple) around and inside the tumor was observed in tumors grown in control (*Left*) compared with selectin-deficient (*Right*) mice. Note that the skeletal muscle near the tumor is stained nonspecifically. Arrows indicate the areas occupied by macrophages. (Scale bar, 100 μm.)

mice 3 or 4 days after injection. Fewer macrophages are present in and around tumors grown in *Rag2*/ELP-null mice (a 4- to 5-fold reduction), and similar results were obtained at later times (data not shown). The numbers of NK cells were low in all sections, and we could not detect significant differences (data not shown).

Fluorescence-activated cell sorter analyses of the circulating F4/80-positive and NK cell populations showed no decreases in single, double-, or triple-selectin-null mice versus *Rag2*-null mice, in accord with previous results (15). We also checked for recruitment of BM-derived endothelial progenitors (using staining for P-selectin). As in the case of integrin-deficient mice discussed above, no recruitment was detected (data not shown). In summary, these results indicate that selectin-dependent recruitment of BM-derived circulating blood cells is important in tumor suppression.

Discussion

The results described here show clearly that certain integrins and all selectins expressed by host cells contribute to the suppression of the growth of transplanted tumors. Because β3-integrins and selectins are expressed by cells of the vessel wall (endothelial cells, pericytes, and smooth muscle cells) and by many circulating blood cells, these results could implicate numerous cell types in tumor suppression.

Prior work has suggested that αv-integrins ($\alpha v\beta 3$ and $\alpha v\beta 5$) could act as negative regulators of angiogenesis (1, 2, 18), and, indeed, we see enhanced vascularization of transplanted human tumors in mice lacking these integrins. The vessels appear normal, with normal investiture by pericytes, as described elsewhere for vessels lacking all αv-integrins (20). The normal association of pericytes and smooth muscle cells with the vessels suggests that failure to recruit these cells is not the reason for the enhanced angiogenesis, as has been suggested (21). An increased number of vessels could contribute to the enhanced tumor growth in integrin-deficient mice. We failed to detect any significant recruitment of BM-derived endothelial progenitors (EPCs) (22–23) to the vessels in any of the mice, although we cannot rule out a low level of recruitment or the possibility that such EPCs might require both integrins and selectins for efficient recruitment. BM-derived cells suppress, not enhance, tumor growth, whereas EPC recruitment might be expected to have an effect opposite to that which we observe. Be that as it may, our experiments clearly implicate other non-vessel wall cells derived from the BM in mediating the tumor-suppressive effects.

Given that all our experiments were conducted in *Rag2*-null mice, there was no involvement of the adaptive immune system (B, T, and NKT cells). However, many other hematopoietic cells could be involved. Many of these cells express β3-integrins and/or L-selectin and selectin ligands (also P-selectin in the case of platelets) and may use P- and E-selectins expressed by endothelial cells as well as integrins during their trafficking and extravasation (24–26). Although we certainly would not wish to rule out platelets, mast cells, eosinophils, or other minor leukocyte populations from consideration, obvious candidate cell types for involvement are neutrophils, monocytes/macrophages, and NK cells.

The numbers of circulating neutrophils in selectin-deficient mice are elevated (15) and are not significantly altered in β3-integrin-null mice. Circulating NK cells are, if anything, elevated in the integrin- and selectin-deficient mice. Histological and immunohistological analyses of the established tumors revealed few neutrophils or NK cells and no obvious differences between mice WT for or deficient in selectins or integrins. However, these results do not rule out a role for these cells, perhaps at an early time during tumor growth or acting in a "hit-and-run" fashion without significant accumulation. Extensive evidence for selectin-dependence of neutrophil traffic exists (24, 26), and some data suggest a role for $\alpha v\beta 3$-integrin in the arrest and extravasation of these cells (27). Neutrophils can exhibit antitumor reactions (28). Recruitment of NK cells has been investigated less completely. However, it has been reported that they express the selectin ligands P-selectin glycoprotein ligand 1 (PSGL-1) and sialyl Lewis X (SLe^X) (29–30) and L-selectin (31), and selectin–ligand interactions likely contribute to their binding to the endothelium. It is well established that NK cells can kill tumor cells (32–34). We have attempted to deplete NK cells from our system, but the results were inconclusive. Such experiments, using well defined genetic backgrounds and appropriate reagents, will be required to investigate in more detail the potential involvement of NK cells and the possible roles of integrins and selectins in their targeting of tumors.

Monocytes/macrophages are known to use $\alpha v\beta 3$-integrin and selectins in their arrest and extravasation at sites of inflammation (35). The same could be true for their infiltration of tumors, and we do detect fewer macrophages infiltrating tumors growing in integrin- or selectin-deficient mice, although the numbers of circulating monocytes/macrophages do not differ among the strains of mice (15). Macrophages have been suggested to be tumoricidal (36–39), although other reports have suggested that they also can contribute to tumor growth (40–42); they probably have both effects (42–46).

To dissect further the possible roles of NK cells and macrophages (and other hematopoietic cells) in the adhesion receptor-dependent tumor suppression that we report here, it will be necessary to perform lineage-specific reconstitution and ablation (genetic or pharmacological). Those experiments should shed light on the role of innate immunity against tumors and the roles of specific adhesion receptors in mediating their effects.

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