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Research Paper

Alternative site of implantation affects tumor malignancy and metastatic potential in mice

Its comparison to the flank model

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Abbreviations: p.i., post injection; PS, physiological solution; SRBC, sheep red blood cells; BSA, bovine serum albumin

Key words: tumor model in mice, dorsal region of foot, cellular influx, immune reaction

MC-C fibrosarcoma and B16F0 melanoma tumors were implanted intradermally in the dorsal region of the foot of mice. Tumor progression was compared to standard implantation in the flank. Although foot tumors only reached 13% (MC-C) and 25% (B16F0) of the mean volume of flank tumors, a more malignant phenotype in terms of histology and survival rate was observed in this type of tumors. Moreover, lung metastases were only detected in hosts bearing foot tumors, in contrast to MC-C and B16F0 populations with tumors growing in the flank. In addition, cellular influx and local immune reaction were higher in the dorsal region of the foot. According to our results, the dermis of the flank allows excessive tumor growth due to its low reactivity. Thus, differences in innate and adaptive immune effectors between the evaluated tumor microenvironments would account for the differences in tumor malignancy. Due to its striking differences with the standard flank inoculation, the tumor implantation model herein introduced could be a valuable tool to study the metastatic potential of different cell lines and the microenvironment components affecting tumor growth.

Introduction

The tumor model most widely applied in experimental oncology involves the inoculation of tumor cells into the flank of mice. However, its physiological relevance is highly questionable and thus it is necessary to find animal models which resemble more closely the physiopathological parameters of human cancer.[1,2] In the case of osteosarcoma, for instance, although several cell lines have been established and characterized, few reliable animal models mimic all the aspects of human cancer, making it difficult to test valuable therapeutic strategies.[3] Since tumor microenvironment plays a key role in cancer development,[4-6] we investigated whether the dermis of a different anatomical site of mice would offer different characteristics than those of the flank. Thus, we chose the dorsal region of the foot and compared it to the flank in terms of cellular influx and reactivity upon antigen injection. Since different levels of response were obtained, we evaluated the growth of two different tumors, MC-C and B16F0, in both anatomic sites. Our results provide a model of tumor implantation in mice that has significant differences with standard inoculation in the flank. We believe that this new model could be a useful tool in the study of tumor progression.

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Results

Cellular influx. Table 1 shows that mononuclear cellular accumulation was significantly higher in the dorsal region of the foot than in the flank of mice.

Immune response to sheep red blood cells. Seven days after immunization with sheep red blood cells (SRBC), sera was obtained and antibody titer was analyzed by hemagglutination assay. Table 2 shows that a significantly higher antibody titer was obtained when SRBC were inoculated in the dorsal region of the foot than in the flank.

Tumor growth. Tumor progression is shown in Figure 1. Tumors grown in the flank reached up to 7,150 (mean ± 287) on day 27 p.i. in the case of MC-C (Fig. 1A), and 8,818 mm^3 (mean ± 2,049) on day 24 p.i. in the case of B16F0 (Fig. 1B). Conversely, tumors grown in the dorsal region of the foot did not exceed 912 mm^3 (mean ± 460) on day 17 p.i. in the case of MC-C (Fig. 1A), and 1,800 mm^3 (mean ± 528) on day 24 p.i. in the case of B16F0 (Fig. 1B), i.e., 6.5% (MC-C) and 25% (B16F0) mean volume of flank tumors.

Mice survival. Mice survival (Fig. 2) was significantly different depending on the implantation site, as demonstrated by Kaplan-Meier analysis. Hosts bearing tumors in the flank lived up to day

Table 1 **Cellular influx**

Implantation site	Total cells (x10^4 cells/ml)
Dorsal region of the foot	202.7 ± 19.73*
Flank	139.0 ± 13.84

Results are the mean of two independent experiments, each group n = 4. *p < 0.05.

Table 2 **Hemagglutination titer**

Injection site	Titer
Dorsal region of the foot	213.3 ± 74.2**,a
Flank	26.7 ± 9.2*
Control	3.4 ± 1.5

Results are the mean of two independent experiments, each group n = 4. **p < 0.01 and *p < 0.05 vs. control; ap < 0.05 between groups.

60 and 36 p.i. for MC-C (Fig. 2A) and B16F0 tumors (Fig. 2B), respectively. In contrast, a poor survival rate was observed in hosts bearing tumors in the dorsal region of the foot: MC-C population decreased dramatically on day 14 p.i., and no mice survived longer than day 17 p.i., whereas B16F0 hosts did not survive after day 24 p.i.

Histology of tumors. Histological study showed significant differences between tumors from both implantation sites. A comparative analysis was carried out with MC-C and melanoma tumors (average 1.2 g) grown in the flank and in the dorsal region of the foot (Fig. 3). MC-C tumors grown in the flank (Fig. 3A) showed characteristic features.[7] There was a predominance of tightly disposed small fusiform cells with oval nuclei and basophilic cytoplasm arranged in a "herringbone" cellular pattern. Mitotic activity was moderate. Intratumoral vascularization was absent, and practically no necrosis was seen. On the contrary, MC-C tumors grown in the dorsal region of the foot (Fig. 3B) were highly vascularized and showed small hemorrhagic foci and no necrotic areas. Gross fusiform cell masses were close to capillary blood vessels. Cellular pleomorphism was predominant and it was characterized by rounded or polygonal big cells with abundant eosinophilic cytoplasm and hyperchromatic nuclei. Giant atypical cells were scarce. Pleomorphic cells were mainly isolated. Inflammatory infiltrates were frequent in these tumors. These features made it difficult to diagnose this tumor as a fibrosarcoma, since it resembled a round cell sarcoma or a malignant angiomatoid fibrous histiocytoma.[27]

In the case of melanoma, tumors growing in the flank (Fig. 3C) showed large and numerous necrotic areas with leukocytic infiltration. Cells were disposed in acines, and intratumoral vascularization was scarce or absent. On the other hand, melanoma growing in the dorsal region of the foot (Fig. 3D) showed a different architecture. Cells were not disposed in acines but distributed in irregular masses. High vascularization, and numerous inflammatory cells and hemosiderin deposits were observed. Another important difference with tumors growing in the flank was the absence of necrotic areas.

Evaluation of metastatic foci. Macroscopic and histological post-mortem examination of serial lung sections from hosts bearing flank MC-C and B16F0 tumors showed neither macroscopic nor microscopic metastatic foci (Fig. 4B and D). However, in order to discard the presence of lung metastases in hosts bearing flank tumors, 6 mm^3 lung pieces were injected intraperitoneally or intradermally in the flank of mice. Twenty days p.i. mice were sacrificed, and checked for the presence of intraperitoneal or intradermal tumor growth. No tumor growth was detected, indicating that no metastatic foci were present in hosts bearing MC-C or melanoma in the flank. Conversely, lung metastases were observed in 100% hosts bearing MC-C or melanoma tumors in the dorsal region of the foot that survived beyond day 14 p.i. (mean). Macroscopic pulmonary metastases per lung were three in average, with a mean volume of 11.7 ± 4.3 mm^3.

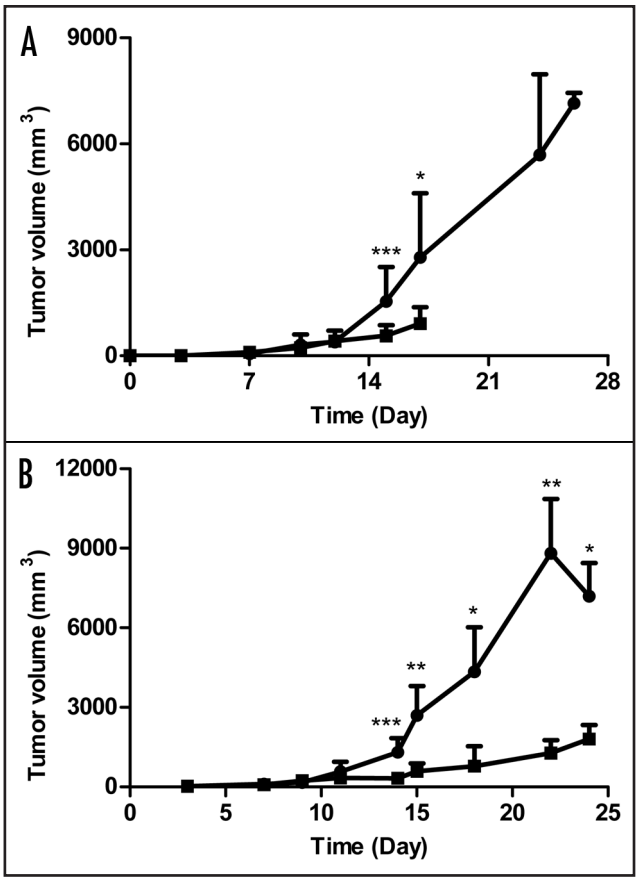

Figure 1. Tumor growth. (A) MC-C and (B) B16F0 tumor progression in the flank (●) and in the dorsal region of the foot (■) is shown. Results are the mean of two independent experiments (each group n = 12). *Points*, mean; *bars*, SD; *p < 0.05; **p < 0.01; ***p < 0.001.

It is worth mentioning that macro and micro lung metastases from MC-C tumors (Fig. 4A) showed histological differences with the primary tumor grown in the dorsal region of the foot (Fig. 3B). In fact, histological study showed similarities to metastases from MC-C tumors grown in the flank (Fig. 3A): fusiform cells tightly disposed and scarce blood capillary vessels. Likewise, histological characteristics of melanoma lung metastases (Fig. 4C) were different than those observed in the primary tumor (Fig. 3D), and closely resembled the architecture of melanoma grown in the flank (Fig. 3C): cells were disposed in acines and a low inflammatory response was observed.

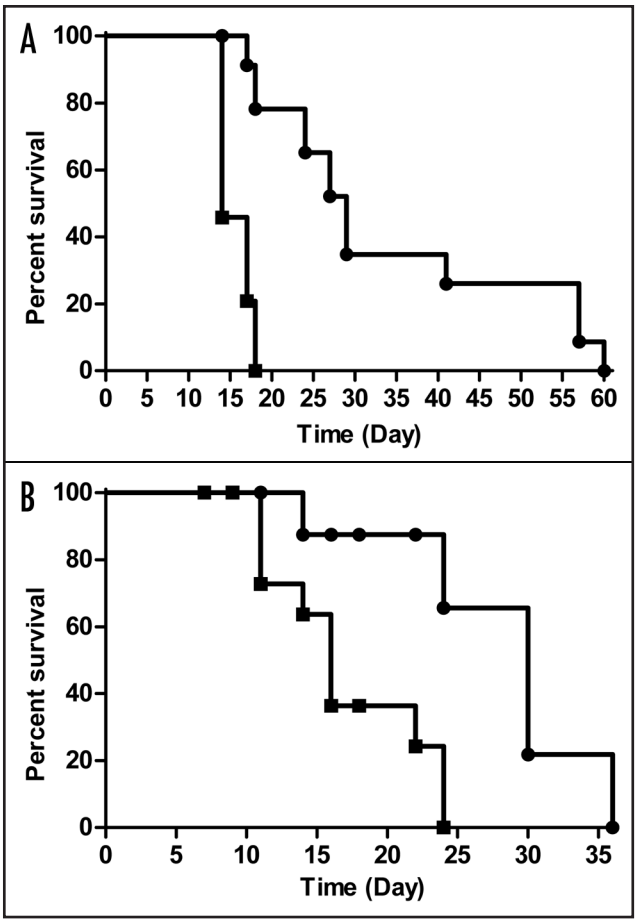

Figure 2. Mice survival. (A) MC-C and (B) B16F0 mice survival in the flank (●) and in the dorsal region of the foot (■) is shown. Survival curves for flank (n = 12) and foot (n = 12) were significantly different for both tumors. Kaplan-Meier analysis for MC-C $p < 0.0001$ and for B16F0 $p < 0.005$.

Discussion

The aim of this study was to compare the characteristics of tumors originated by the standard procedure of inoculation in the flank and tumors grown in the dorsal region of the foot. Microenvironment is a key factor in tumor progression. Seftor et al.[5] demonstrated that poorly aggressive melanoma cells acquired a vasculogenic phenotype when they were exposed to a microenvironment preconditioned by aggressive metastatic melanoma cells. The opposite effect has also been studied long ago by Illmensee et al.[8] who showed that teratocarcinoma cells could be normalized by the blastocyst environment. More recently, melanoma metastatic cells could be reverted to their original cell type, the neural-crest-derived melanocyte, using an embryo chick model.[9] Thus, we investigated whether there were differences between the dermis from the flank and from the dorsal region of the foot that could generate tumors of different malignancy in mice.

Cellular influx and local immune response assays showed that the dorsal region of the foot had higher reactivity. Both inoculation sites were tested with two different tumors, fibrosarcoma and melanoma, and results obtained exhibited a similar pattern; tumors grown in the dorsal region of the foot showed higher malignancy than flank tumors, in agreement with survival rate and histological studies. Moreover, lung metastases were only observed in hosts bearing MC-C and melanoma tumors in the dorsal region of the foot, and no lung metastases were detected in hosts bearing these tumors in the flank, even though this group showed longer survival rate. Histological features of lung metastases both from MC-C and melanoma differed from those of the primary tumor growing in the dorsal region of the foot, suggesting that a reversion to a more differentiated phenotype similar to that of flank tumors has occurred. This would spontaneously confirm that tumor malignancy would be related to the microenvironment response, either for the benignization or malignization of its phenotye. Using a variety of model systems, other authors have demonstrated this phenomenon. Okada et al.[14] reported that stroma promotes the conversion of colonic adenoma cells to adenocarcinoma cells, whereas Mueller & Fusenig [15] concluded that stroma activity modulates tumor phenotype. Likewise, Kenny & Bissell[16] and Maffini et al.[17] demonstrated that tissue microenvironment plays a critical role in carcinogenesis using a breast tumor model.

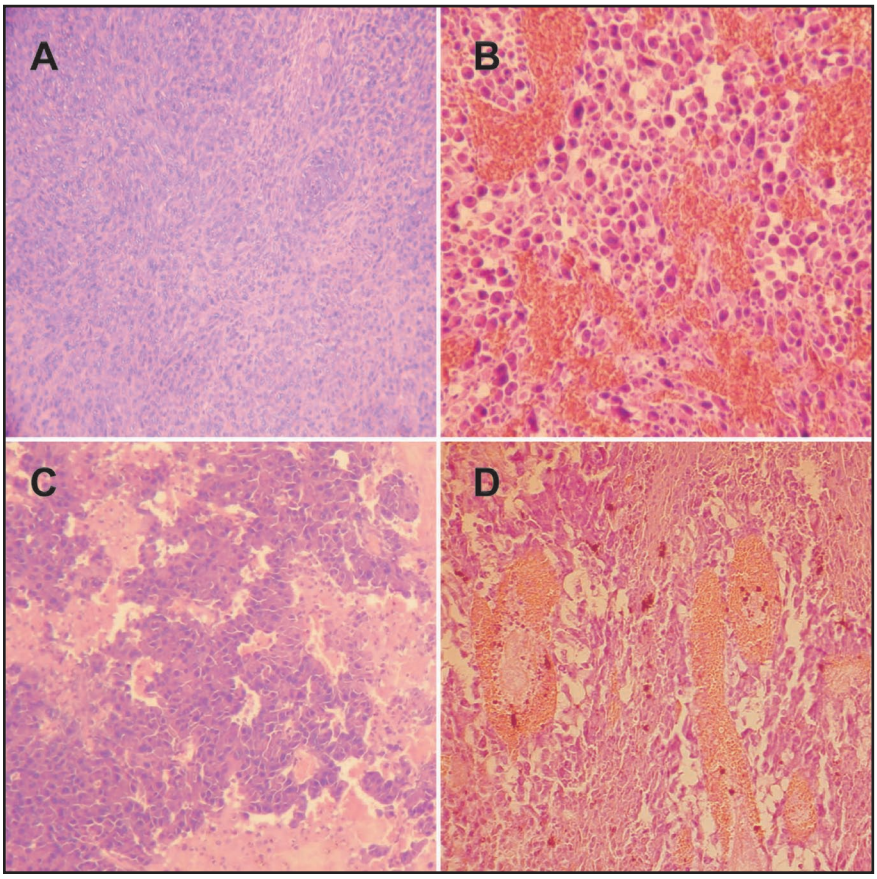

Figure 3. Histological analysis of tumors. Sections stained with hematoxylin-eosin from MC-C and B16F0 growing in the flank (A and C), respectively, and in the dorsal region of the foot (B and D), respectively, are shown. Original magnification 10x.

The model of tumor implantation in the dorsal region of the foot herein proposed could be a useful tool to study spontaneous metastases of tumors that would not generate metastases if they grow in the flank. The flank of mice seems not to be a suitable microenvironment for tumor cells to display their whole biologic potential. For instance, in several osteosarcoma cell lines,[10,11] orthotopic inoculation had to be performed to find a model that closely mimic clinical progression, since no metastases were observed when cells were injected subcutaneously into the flank.

In addition, our results provide further evidence of a correlation between tumor malignancy and local immune response at the site of implantation, in which higher immune response would favor tumor growth. Results from tumor inoculation in the dorsal region of the foot agree with reports from other authors, who showed that the immune reaction may enhance rather than inhibit neoplastic growth.[12,13] Therefore, differences in innate and adaptive immune effectors between the evaluated microenvironments could account for differences in tumor malignancy. Many malignancies arise from areas of inflammation, simply as part of the normal host response.[24] Our study proposes that the dorsal region of the foot has higher reactivity due, in part, to higher cellular influx. This area has higher capillary density than the flank as shown by ex vivo analyses of the dermis from both sites (data not shown). As already described, capillary blood vessels would facilitate leukocyte recruitment to the site of tumor implantation,[25] enhancing the inflammatory component of the dorsal region of the foot. Thus, inflammatory cells would provide an attractive environment for tumor growth, enhancing genomic instability and promoting angiogenesis.[24,28] Moreover, the dorsal region of the foot displays a higher immune response upon antigen injection than the flank. This could be attributed to a higher antigen retention in the foot of mice, as demonstrated by Tew et al.[26] Therefore, the higher inflammatory and immune response naturally displayed by the dorsal region of the foot could enhance tumor malignancy.

Mouse models used in the study of human cancer have several limitations, particularly as regards the standard subcutaneous injection.[1,2] One of the most significant differences between human cancer and tumors growing in the flank of mice is related to the volume that the tumor can reach. Subcutaneous growth in the flank often results in tumor masses that reach approximately the weight of its host. During our study we observed that MC-C fibrosarcoma may reach 11.04 ± 6.1 g, and B16F0 melanoma, 6.8 ± 2.13 g, i.e., 55% and 34% the average weight of the host, respectively. Thus, conclusions about tumor growth and any potential implication for cancer patients must be evaluated very carefully. We believe that one of the reasons for the excessive but slow growth of tumors in the flank of mice might be related to the fact that it is a site of poor immune response. Further studies on the immune response in both anatomic sites are currently being carried out.

The model herein introduced could be an alternative to the standard inoculation of tumors into the flank of mice. Equally easy to handle, this new model might be useful to study tumor progression and metastases.

Collectively, our results might suggest that the degree of malignancy is not an inherent phenomenon of the tumor, but a response to the reactivity of the microenvironment.

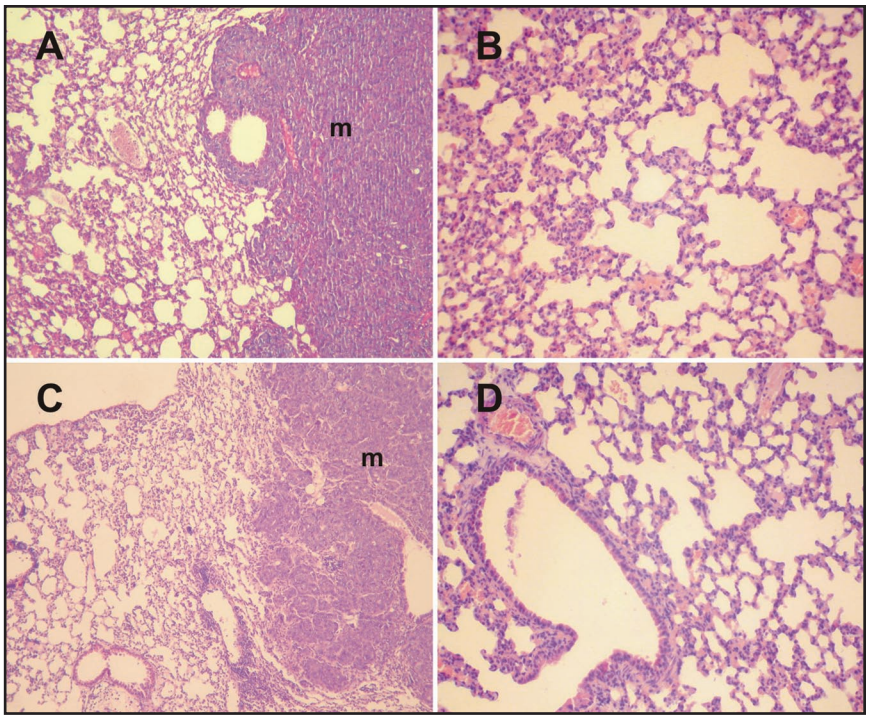

Figure 4. Histological analysis of lungs. Sections stained with hematoxylin-eosin from lungs of host bearing MC-C and B16F0 melanoma growing in the dorsal region of the foot (A and C), respectively, and in the flank (B and D), respectively are shown. Post-mortem examinations revealed pulmonary metastases (m) when the primary tumor grew in the dorsal region of the foot (A and C), and no lung metastases in lungs from mice bearing tumors in the flank (B and D). Original magnification 20x.

Materials and Methods

Animals and tumor cell lines. BALB/c and C57BL/6 mice, 6–8 weeks old, were used. MC-C, a murine fibrosarcoma induced by methylcholanthrene,[18] and B16F0 melanoma cell line kindly provided by the Laboratorio de Oncología Molecular, Departamento de Ciencia y Tecnología, UNQ, were used. These tumors differ in their aggressiveness and immunogenicity. B16F0 melanoma is poorly or non-immunogenic and recidivates after removal.[21,22] MC-C tumor, on the other hand, has the characteristics of a non-infiltrating slow growing fibrosarcoma.[7,23] It is highly immunogenic and, unlike B16F0 melanoma, it never recidivates after removal.

Cellular influx. A sterile nylon sponge fragment of 1 mm^3 was implanted subcutaneously[7] either into the flank or the dorsal region of the foot of BALB/c mice. The sponge was removed four days post-implantation, and repeatedly compressed in 0.1 ml physiological solution (PS). Mononuclear cells from the resulting suspension were counted in a Neubauer's chamber.

Immune response to sheep red blood cells. Sheep red blood cells (SRBC) (Laboratorio Alfredo Gutiérrez) were washed three times with 5 vol PS, pH 7.2, before use. Then, they were suspended in PS 0.2% BSA. A 40% v/v suspension was used for mouse immunization. An aliquot of 50 µl of this suspension was injected intradermally either

in the flank or the dorsal region of the foot. Seven days p.i., blood was collected from the retro-orbital plexus and sera were obtained for the hemagglutination assay.[19] The test was performed in a 96 well plate. Individual mouse sera were serial diluted in PS 0.2% BSA to a final 50 µl volume. Dilutions ranged from 1:2 to 1:2048. Then, 50 µl 0.1% v/v SRBC suspensions were added to each well. The plate was incubated overnight at room temperature. Hemagglutination titer of each serum was recorded as the reciprocal of the highest dilution in which agglutination was macroscopically observed. Sera from non-immunized mice were used as controls.

Tumor growth. MC-C and B16F0 tumors were implanted in BALB/c and C57BL/6 mice, respectively. Tumors (6 mm^3) were injected intradermally either in the right flank or in the dorsal region of the foot. Tumor volume was calculated according to the formula of Attia and Weiss:[20] tumor volume = 0.4 ab^2, where a and b are the longest and the smallest diameters, respectively. Mice survival was recorded.

Evaluation of metastatic foci. Mice were anesthetized with ether and sacrificed by cervical dislocation. Lungs were removed and surface metastatic nodules (diameter ≥0.1 mm) were counted under a dissecting microscope. Lungs were then processed for the histological study.

Histological study. Tumors were removed and fixed in 10% formaldehyde phosphate-buffered saline, pH 7, and embedded in paraffin. Serial sections (3–5 µm) were obtained and stained with hematoxylin and eosin.

Statistical analysis. Data were analyzed by ANOVA and Student's t test. Differences between means were considered significant when $p < 0.05$. Results are expressed as means ± SD. For the Kaplan-Meier analysis, survival differences between groups were evaluated using Logrank test.

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