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Bone marrow cells participate in tumor vessel formation that supports the growth of Ewing's sarcoma in the lung

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Abstract

An MHC-mismatch bone marrow (BM) transplant Ewing's sarcoma mouse model was used to investigate whether BM cells participate in the vessel formation that support Ewing's sarcoma lung metastasis. BM cells from H-2K^{b/d} donor mice were transplanted into sublethally irradiated H-2K^d recipient mice. Donor BM cells were identified using the H-2K^b marker. Engraftment was confirmed by identifying the H-2K^b IL-1 β -type specific polymorphism. After engraftment highly lung metastatic TC71-PM4 cells were injected intravenously. Mice were sacrificed 10 weeks after tumor cell injection. Hematoxylin-and-eosin staining was performed to identify lung metastatic foci. These tumors were then evaluated using immunohistochemical analysis. H-2K^b-positive cells were found in lung metastases but not in normal lung, liver or spleen tissues. Injection of CM-Dil-labeled BM cells into tumor bearing and control mice showed that nonspecific organ migration occurred at 24 h, but that these cells were absent 1 week later in control mice. These data suggest that the migration of the H-2K^b BM cells to lung nodules was specific because these cells were observed 14 weeks after transplantation. Co-localization of H-2K^b and CD31 or VE-Cadherin demonstrated that some endothelial cells were BM-derived. Co-localization of H-2K^b and Desmin, smooth muscle actin (α -SMA) or PDGFR- β indicated that a fraction of pericytes was also BM-derived. These results suggest that BM cells participate in the vascular formation that supports the growth of Ewing's sarcoma lung metastases. BM cells migrated to the metastatic tumor and differentiated into endothelial cells and pericytes. These data indicated that targeting this process may have therapeutic potential.

Keywords

Bone marrow cells; Vasculogenesis; Metastasis; Ewing's sarcoma; Neovascularization

Introduction

Tumor metastasis is a major cause of death among cancer patients [1], thus, treatments that target the metastatic process are critical to improve cancer cure rates. When metastatic cells spread to distant organs, their growth and survival depends on the development of a vascular system [2]. Understanding the processes and mechanisms that contribute to the formation of these new blood vessels can help us identify therapies that can interfere with or block these processes, thereby impeding growth and inducing regression.

Ewing's sarcoma is a vascular tumor that metastasizes to the lungs. The survival rates of patients with lung metastases is <20%, despite multiple alterations in the chemotherapy regimens over the past 20 years [3–7]. It is unlikely that patient outcomes will improve without the introduction of newer targeted therapies. Understanding how tumor cells manipulate the microenvironment to their advantage and which host cells contribute to the formation of tumor vessels may allow us to develop therapies that disrupt this interaction.

We previously demonstrated that bone marrow (BM) cells participate in the vascular expansion that supports the growth of Ewing's tumors in the leg [8–11]. BM cells migrated to the tumor area and were incorporated into the endothelial lining of functional tumor neovessels [10]. We further showed that Ewing's tumor vessels are a mosaic of locally derived and BM-derived endothelial cells and pericytes [10]. Interfering with this process and inhibiting BM cell migration to the tumor area reduced tumor vessel expansion and inhibited tumor growth and metastasis [11–15]. Together, these data suggest that therapy aimed at disrupting the participation of BM cells in tumor vessel formation may have therapeutic benefit against primary Ewing's tumors and also retard future metastasis. However, whether BM cells also participate in the tumor vessel formation that supports the growth of Ewing's sarcoma metastases in the lung has not been elucidated. This is a critical question for determining the potential for such a therapeutic approach for patients with microscopic metastases or even small metastatic foci at the time of diagnosis. To tackle this question we created a highly metastatic Ewing's sarcoma subclone, TC71-PM4, by cycling TC71 cells in mice. TC71-PM4 tumor cells preferentially colonize the lung and consistently induced high numbers of lung tumor nodules. Using our major histocompatibility complex (MHC)-mismatch BM transplantation model to track the migration and differentiation of BM cells [8], we demonstrated that BM cells also migrate to TC71-PM4 lung tumors and contribute to the pool of endothelial cells and pericytes that make up these tumor vessels in the lungs. These findings indicate that BM cells and the process of vasculogenesis are involved in the development of new vasculature that supports the growth of Ewing's sarcoma lung metastases as well as primary tumors in the bone.

Materials and methods

Human Ewing's sarcoma cell lines

TC71 human Ewing's sarcoma cells were cultured in Eagle's modified essential medium with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1× nonessential amino acids, and 2× minimal essential medium vitamin solution (Life Technologies, Inc., Grand Island, NY). All cells were negative for Mycoplasma, as determined using a Mycoplasma Plus polymerase chain reaction (PCR) primer set (Stratagene, La Jolla, CA).

TC71-PM4 cells were derived from TC71 parent cells by repeated cycling through mice, as previously described [16]. In brief, TC71 cells were intravenously injected into nude mice. Lung nodules were detected 10 weeks later in 25% of mice. These tumors were removed, digested, resuspended, and then reinjected to yield TC71-PM cells. This process was

repeated 2 additional times, yielding the TC71-PM4 subline. TC71-PM4 cells have high lung metastasis potential, with 67% of mice developing lung tumors 10 weeks after intravenous injection.

MHC-mismatched BM transplantation model

Male 8–13-week-old nude BALB/cAnN mice with the MHC strain-specific surface antigen d (H-2^d) and C57BL × BALB/c F1 (CB6 F1) mice (H-2^{b/d}) were purchased from the National Cancer Institute. The mice were maintained in a specific pathogen-free animal facility approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with the current regulations and standards of the US Department of Agriculture, the Department of Health and Human Services, and the National Institutes of Health. The animal experiment protocols were approved by the Institutional Animal Care and Use Committee.

CB6 F1 mice (H-2^{b/d}) were used as the BM donors, and BALB/cAnN mice (H-2^d) were used as the BM recipients. This allowed us to track BM cells by detecting H-2K^b antigen in H-2K^d recipient mice. BM transplantation was performed as previously described [8]. In brief, recipient mice underwent whole-body irradiation with 9.0 Gy using an external cesium source (¹³⁷Cs Mark 1 irradiator; J. L. Shepherd & Associates, Glendale, CA). Freshly isolated BM cells ($3 \times 10^6/200 \mu\text{L}$) flushed from the femurs of CB6F1 mice were injected into the lateral tail veins of nude BALB/cAnN mice within 2 h after irradiation. Engraftment was confirmed using strain-specific H-2 surface antigens for donor (H-2^b) interleukin-1 β (IL-1 β) in the spleen and blood.

PCR analysis of DNA polymorphism with IL-1 β

DNA was extracted from mouse spleens, blood, and livers using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. Engraftment of donor BM cells was verified by PCR analysis of a polymorphic microsatellite region of the murine IL-1 β gene. The primer sequences were 5'-CCAAGCTTCCTTGTGCAAGTA-3' and 5'AAGCCCAAAGTCCATCAGTGG-3'. The oligonucleotides were provided by Sigma-Genosys (St. Louis, MO). Amplification was performed with an initial step of 1 min and 30 s of denaturation at 95°C; 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of elongation at 72°C; and one cycle of 30 s of annealing at 57°C and 7 min of elongation of 72°C. The PCR conditions were 50- μL total volume with 200 ng of DNA as template, 0.1 μM primers, 0.4 mM deoxynucleotide triphosphate, 1 $\times$ PCR buffer with magnesium, and 2.5 units of Taq DNA polymerase (Roche, Nutley, NJ).

Lung colonization technique to induce TC71-PM4 pulmonary tumors in mice

Four weeks after BM transplant, 2×10^6 of TC71-PM4 cells were intravenously injected into recipient nude mice. Mice were euthanized with CO₂ 10 weeks after tumor cell injection. The TC71-PM4 lung nodules were analyzed by H&E staining to evaluate the metastatic potential. Frozen lung sections were examined using immunofluorescent staining to identify BM-derived cells, endothelial cells and pericytes by different markers.

Subcutaneous tumor mouse model

Eight weeks after BM transplant, 3×10^6 of TC71 cells were subcutaneously injected into recipient nude mice. Mice were killed 4 weeks after tumor cell injection, and their subcutaneous tumors were removed for immunofluorescent staining.

Immunofluorescent staining in tumor tissues

Rat anti-mouse CD31 and biotinylated anti-mouse H-2K^b antibodies were purchased from BD Biosciences Pharmingen. (San Diego, CA). Desmin and α -smooth muscle actin (α -SMA) antibodies were obtained from Abcam, Inc. (Cambridge, MA). VE-Cadherin, PDGFR- β and F4/80 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The frozen lung sections were fixed with acetone and chloroform. Incubation in 4.5% fish gelatin for 20 min was used to block nonspecific proteins. Expression of CD31 was detected using rat anti-mouse CD31 as the primary antibody and goat anti-rat TexasRed (Jackson ImmunoResearch, West Grove, PA) as the secondary antibody. For confocal microscopy, anti-rat Cy-5 was used as the secondary antibody. For double fluorescence staining, the sections were first incubated with an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA). H-2 K^b was then used as the primary antibody and streptavidin-FITC (BD Biosciences) as the secondary antibody.

Tracking BM cells labeled with CM-Dil fluorescent dye

To determine whether BM cells specifically migrate to the metastatic tumor microenvironment, rather than non-specific to all organs, BM cells labeled with CM-Dil were used for tracking. Mouse BM cells were obtained from CB6F1 mouse femurs. The cells were washed in sterile phosphate-buffered saline, spun at 1,500 rpm for 5 min, resuspended at a density of 1×10^6 cells/mL, and stained with CM-Dil fluorescent dye at a concentration of 5 μ L/mL (Vybrant Cell-Labeling Solution; Molecular Probes, Carlsbad, CA) to track their migration. The cells were then incubated at 37°C for 15 min, washed twice with phosphate-buffered saline, and injected intravenously into mice (3×10^6 stained cells in 100 μ L per mouse). Group A included control mice without tumors. Group B mice had lung tumors that had been induced by intravenously injecting them with TC-PM4 cells 10 weeks prior to the injection of the CM-Dil-labeled BM cells. The mice were killed 1 or 7 days later. The lungs, livers, and spleens were removed, frozen, and analyzed with a Zeiss fluorescence microscope (Carl Zeiss MicroImaging, Thornwood, NY) to determine the presence and location of CM-Dil⁺ cells.

Results

Confirmation of H-2K^{b/d} donor BM cell engraftment using IL-1 β strain-specific polymorphism

To confirm engraftment of transplanted H-2K^{b/d} BM cells, we assessed a strain-specific DNA polymorphism in the IL-1 β gene [17]. The recipient nude mice were H-2K^d, whereas the donor mice were H-2K^{b/d}. Therefore, successful engraftment would result in the identification of both H-2K^b and H-2K^d in recipient chimeric mice. In Fig. 1, H-2K^d strain-specific IL-1 β DNA is shown in the top band (lanes 1–5), and H-2K^b IL-1 β DNA is shown in the bottom band (lanes 1 and 2). DNA extracted from the spleens (lane 1) and blood (lane 2) of transplanted mice demonstrated both the H-2K^b and H-2K^d IL-1 β polymorphisms, whereas DNA extracted from the spleens (lane 3), livers (lane 4), or blood (lane 5) of non-transplanted nude mice demonstrated only the H-2K^d IL-1 β polymorphism. These data confirm that engraftment with H-2K^{b/d} BM cells was successful.

BM cell migration into TC71-PM4 lung tumor nodules and differentiation into endothelial cells and pericytes

Nude mice were transplanted with H-2K^{b/d} BM cells, as described above. Engraftment was confirmed 1 month later, and mice were intravenously injected with TC71-PM4 cells. Ten weeks after tumor cell injection, the mice were killed and their lungs were removed and examined using H&E staining and immunohistochemical analysis. Lung tumor nodules were

detected in 6 of 10 mice (60%). H-2K^b staining was used to identify BM-derived cells. Sequential tumor slicing allowed us to use H&E staining (Fig. 2A) to assess the location of these BM-derived cells. H-2K^b-positive cells (green) were detected in lung tumors, indicating that these cells were BM-derived and had migrated from the BM to the metastatic tumor (Fig. 2B). These H-2K^b-positive cells were located inside the tumors but were not found in the normal surrounding lung tissue. To determine the location of these BM-derived cells with respect to tumor vessels, we stained tumor sections with anti-CD31 to identify microvessels (Fig. 2C). Merging the two images revealed that the migrated BM-derived cells were closely associated with, as well as distinct from, the CD31⁺ microvessels (Fig. 2D). The yellow areas (arrow) in these merged images indicate migrated cells that had been integrated into the tumor vessel. Nuclear staining confirmed that these BM-derived cells were within the tumor (Fig. 2E). To further identify vascular endothelial cells, another endothelial cell marker, VE-Cadherin, was used for immunofluorescent staining in addition to CD31. (Fig. 3A right panel for VE-Cadherin, left panel for CD31). These high power magnification (40×) confocal microscopy images show colocalization (yellow) of cells that express both H-2K^b (green) and CD31 or VE-Cadherin (red), indicating that these are BM-derived endothelial cells. There were also CD31⁺ or VE-Cadherin⁺ cells which did not express H-2K^b (red) indicating that these endothelial cells were derived from the local population of cells. Finally there were H-2K^b⁺ cells that were CD31⁻ or VE-Cadherin⁻ (green) indicating that these BM cells did not contribute to the endothelial cell population of the tumor vasculature.

To determine whether BM-derived cells contributed to pericyte formation, we analyzed the tumor sections using immunofluorescent staining for H-2K^b and the pericyte differentiation markers α -SMA, Desmin and PDGFR- β , (Fig. 3B). Similar to what was observed for endothelial cells, the pericyte population was composed of both BM-derived and locally derived cells. H-2K^b cells expressing Desmin (Fig. 3B, upper panel, yellow) α -SMA (Fig. 3B, middle panel, yellow) or PDGFR- β (Fig. 3B bottom panel, yellow) were detected in the tumor vasculature, indicating that a fraction of migrated BM cells had differentiated into pericytes/vascular smooth muscle cells. Together, these data indicate that after migration to the tumor area, BM cells differentiate into both endothelial cells and pericytes.

To quantify the percentage of H-2K^b positive BM-derived cells that differentiated into endothelial cells or pericytes, the computer image software Simple PCI (Hamamatsu Inc.) was used to quantify the total H-2K^b positive area, and the colocalization area for H-2K^b and CD31 or Desmin in at least five random microscope fields (10× magnification). The data demonstrated that $9.1 \pm 2.5\%$ of H-2K^b positive BM cells differentiated into the endothelial cells, while $15.9 \pm 4.7\%$ of H-2K^b positive BM cells differentiated into pericytes. We also quantified the CD31⁺/H-2K^b⁺ cells (endothelial cells derived from BM cells), the CD31⁺/H-2K^b⁻ cells (endothelial cells derived from local tissue), the Desmin⁺/H-2K^b⁺ cells (pericytes derived from BM cells) and the Desmin⁺/H-2K^b⁻ cells (pericytes derived from local tissue). The results indicated that $10.3 \pm 2.1\%$ of endothelial cells and $21.5 \pm 5.0\%$ of pericytes were derived from BM cells. The ratio between endothelial cells and pericytes differentiated from BM is 1:2 (10.3%: 21.5%). The endothelial cells and pericytes derived from local pulmonary tissues were $89.7 \pm 2.2\%$ and $78.3 \pm 4.9\%$, respectively. The ratio between endothelial cells and pericytes recruited from pulmonary tissues is 1: 0.9 (89.7%: 78.3%). We have previously shown that not all BM cells that migrate into the tumor participate in tumor vessel formation [10]. These BM-derived cells were found within the tumor parenchyma and were CD31⁻/F4/80⁺ indicating that these were monocyte/macrophages. We therefore also quantified the colocalization of F4/80 and H-2K^b positive cells in the tumors. Approximately $22.4 \pm 7.3\%$ of BM-derived cells developed into tumor associated monocyte/macrophages. This correlates with our previous data with subcutaneous TC71 tumors [10].

Migration of BM cells into lung metastases is specific

To determine whether the migration of BM cells to TC71-PM4 lung metastases was secondary to random circulation and retention, we examined normal lung, liver, spleen, and subcutaneous tumor tissues for the presence of H-2K^b BM-derived cells. For these experiments, mice underwent transplantation as described above. Tumor cells were injected intravenously (to induce lung tumors) and subcutaneously (to induce primary tumors) 4 and 8 weeks after the BM transplant respectively. Unlike lung tumor nodules, H-2K^b cells were not detected in normal lung, liver, or spleen tissues (Fig. 4A). By contrast, subcutaneous tumors contained H-2K^b-positive BM cells, similar to what was observed in lung nodules (Fig. 2). Colocalization revealed H-2K^b⁺/CD31⁺ cells (yellow, arrow in Fig. 4A) in the subcutaneous tumor only. These data indicate that BM cell retention and differentiation was specific to subcutaneous tumors and lung metastases. This was further confirmed by intravenously injecting CM-Dil-labeled BM cells into mice with or without tumors. Groups of mice were killed at different time points, and their organs were removed and examined for the presence for CM-Dil-positive cells. CM-Dil is stable for at least 1 month, allowing us to track cell migration and retention for an extended time period. Labeled BM cells were identified in the livers, spleens, and lungs of control mice and tumor-bearing mice 24 h after intravenous injection (Fig. 4B, first and third panels). However, after 1 week, no labeled cells were detected in the lungs of mice without tumors (Fig. 4B, second panel). By contrast, labeled BM cells (21 ± 6 per low power field) were observed in the lungs of mice with lung nodules but not in their livers or spleens (Fig. 4B, fourth panel). These data suggest that while non-specific organ migration of BM cells occurs, these cells are cleared within 1 week and only retained in the organ where the tumor is. This is consistent with the results shown in Fig. 2. H-2K^b-positive BM cells were only detected in lung tumors 10 weeks after tumor cell injection and 14 weeks after BM cell transplant, suggesting that the migration and retention of BM cells to the lungs is a specific event due to the tumor microenvironment.

Discussion

The metastatic process is complex, involving a series of sequential and inter-related steps. After metastatic cells travel from the primary site to distant organs, the neovasculature must be established to support the cell growth [2]. We and other investigators have shown that BM-derived cells play an important role in the growth and metastasis of primary tumors, including Ewing's sarcoma [8,10,11,18]. Clinical studies have also shown that high levels of circulating vascular endothelial growth factor receptor 2 (VEGFR2)⁺ BM-derived progenitor cells are correlated with metastatic disease in patients with pediatric solid tumors [19]. The role of BM cells in the formation of the vascular network that supports the growth of metastases in distant organs has been less well defined. The data presented here demonstrate that BM cells and the process of vasculogenesis contribute to the expansion of tumor vessels in Ewing's sarcoma lung tumors as well as the primary subcutaneous tumors. Using our MHC-mismatched BM transplant model [8] and a high lung colonization TC71 Ewing's sarcoma subclone (TC71-PM4), we demonstrated that BM cells migrate to the lungs, specifically to the tumor area, and differentiate into both endothelial cells and pericytes that comprise the tumor vessels. The BM-derived cells were seen only in the tumor and not in the surrounding normal lung, liver and spleen (Fig. 2 and 4A). While the BM-derived endothelial cells and pericytes comprised less than 20% of the cells that contributed to the formation of the tumor vasculature, we have shown the importance and critical nature of these cells in our primary subcutaneous TC71 tumor model [12,13,15] where only 10% of the vessels contained BM-derived cells [8]. Inhibiting the migration of these cells into the tumor severely retarded tumor growth. Similarly we showed that transplanting mice with MEKK3-deficient BM cells which can not differentiate into endothelial cells or pericytes resulted in small, avascular tumors [15]. Taken together, these experiments indicate that BM

cells play a critical role in tumor vessel expansion despite the fact that the majority of tumor vessel endothelial cells and pericytes are locally derived. Furthermore, these cells may provide a way for tumors to circumvent the effects of anti-angiogenic therapy such as Avastin. We have shown that BM cells can form tumor vessels in the absence of VEGF and rescue tumor growth using our subcutaneous TC71 Ewing's sarcoma mouse model [13]. This same mechanism may also help the pulmonary tumors circumvent the effects of therapy and rebound.

Specific recruitment and retention of BM cells to the tumor area was further demonstrated using CM-Dil-labeled BM cells to track their migration pattern after intravenous injection. BM cells were observed in the lungs, livers, and spleens of both tumor-bearing and control mice 24 h after injection, but these cells were only present in the lungs of tumor-bearing mice 1 week after injection. These findings suggest that non-specific migration into multiple organs occurs during the first 24 h after injection but that the BM cells are only retained in the tumor-bearing organ, where they differentiate into cells that participate in tumor vessel formation. In our transplant experiments the BM cells were injected intravenously, however, we waited 4 weeks after transplantation to inject tumor cells. Therefore, any residual BM cells that might have extravasated into the lungs would have been gone at the time of tumor cell injection. Together, these data suggest that the organ microenvironment is altered by tumor cells to attract and retain BM cells for the purpose of tumor vessel formation. Indeed, we previously showed that TC71 cells overexpress the VEGF₁₆₅ isoform, which can mobilize and recruit circulating stem and progenitor cells [11]. We further demonstrated that this isoform is responsible for the chemotaxis of BM cells to primary Ewing's sarcoma tumors [12,14]. Similar to the TC71 cells, TC71-PM4 cells overexpress VEGF₁₆₅ (data not shown), indicating that these cells can produce a cytokine that stimulates the specific migration of BM cells into the tumor area, which in the case of the TC71-PM4 cells is the lungs. An alternative interpretation of our data is that the BM cells migrated to the lungs at the time of transplant, resulting in a favorable metastatic niche for the tumor cells when they were subsequently injected. We do not favor this interpretation as we showed that BM cells transiently migrated to the spleen and liver and yet no metastases developed. Nevertheless we can not exclude this as a possibility. It may also be a combination of both migration into the lung before colonization to prepare the microenvironment and migration after colonization to help form the needed vasculature for tumor growth. Our data demonstrated that BM cells participate in tumor vessel formation regardless of when they migrated.

BM cells express VEGFR2. Therefore, the presence of VEGF₁₆₅ in the lung microenvironment will result in the chemotaxis and retention of BM cells in the lungs. Indeed, in our previous studies, VEGFR2⁺ BM cells differentiated into endothelial cells and pericytes in the subcutaneous tumor vessels [10]. This finding supports our hypothesis that VEGF₁₆₅ plays a critical role in the chemoattraction of BM cells to the primary and metastatic tumor microenvironments. In our previous studies, we also demonstrated that interfering with the migration of bone marrow cells to the tumor area resulted in decreased tumor vascularity and retarded primary tumor growth [13,14,20]. In addition to VEGF₁₆₅, other molecules may also be involved in BM cells recruitment to the tumor area. Stromal cell-derived factor-1 (SDF-1) is a chemoattractant for human CD34⁺ hematopoietic progenitor cells and has also been shown to play a role in the recruitment of bone marrow-derived cells [21,22]. Our previous studies demonstrated that SDF-1 stimulated the migration of bone marrow cells and enhanced the growth of VEGF-inhibited tumors with no increase in VEGF₁₆₅ [13]. Hypoxia-induced lysyl oxidase (LOX) is another critical mediator in bone marrow cell recruitment, responsible for the formation of the premetastatic niche [23]. Tumor hypoxia may stimulate VEGF, SDF-1 and LOX which in turn will result in the recruitment of BM cells into the tumor area and then differentiation into endothelial cells and pericytes which help expand the tumor vasculature.

Other studies have shown that BM progenitor cells contribute to the angiogenic switch in mouse lung metastases [18,24] and that mobilization of these cells promotes the metastatic process. The studies presented here are the first to demonstrate that BM cells and the process of vasculogenesis contribute to the formation of the tumor vascular network that supports the growth of Ewing's sarcoma lung metastases. Together, these data indicate that agents that target this process and this population of cells may have therapeutic potential. Targeting this cell population may retard tumor vessel formation, which would negatively affect the growth of the metastatic tumor by compromising the delivery of oxygen and nutrients to tumor cells, which are necessary to sustain tumor growth. Incorporating such agents into the treatment schema for patients with Ewing's sarcoma, particularly for those who are at high risk for developing metastasis or those that already present with metastasis, may result in improved outcomes. Because the long-term survival of patients with high-risk and metastatic Ewing's sarcoma has not changed for over 20 years, considering such novel therapeutic approaches is warranted.

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Fig. 1.

Detection of H-2K^b-specific IL-1 β polymorphism in the spleens and peripheral blood of transplanted mice. A strain-specific DNA polymorphism in the IL-1 β gene was assessed in H-2K^{b/d}-transplanted and H-2K^d non-transplanted mice. The *top band* indicates the recipient H-2K^d strain, and the *bottom band* indicates the H-2 K^b donor strain. *Lanes 1 and 2* correspond to DNA extracted from the spleens or blood of the recipient H-2K^d mice transplanted with H-2K^{b/d} BM cells from donor mice. The presence of both *bands* indicates that the cells are of donor origin. *Lanes 3–5* correspond to DNA extracted from the spleens, livers, or blood of non-transplanted H-2K^d mice. *Lane 6* is the PCR-negative control

Fig. 2. H-2K^b donor BM cells migrate to lung tumors and differentiate into endothelial cells in the tumor vessel network. TC71-PM4 lung tumors from H-2K^d mice transplanted with H-2K^{b/d} BM cells were examined using H&E staining (**A**) and corresponding sections using immunohistochemical analysis (**B–E**). Images (**A–E**) are 10× magnification. (**B**) Identification of H-2K^b positive cells (*green*) was used to distinguish cells of donor origin from cells of the host (H-2K^d). (**C**) CD31 staining (*red*) was used to identify endothelial cells and tumor vessels. The merged images (**D**) show that a portion of cells expressed both markers (*yellow*), indicating that endothelial cells were BM-derived. (**E**) DAPI nuclear staining was used to identify individual tumor cells

Fig. 3. H-2K^b positive BM cells differentiate into endothelial cells and pericytes in the tumor vessel network of TC71-PM4 lung tumors. The tumors from H-2K^d mice transplanted with H-2K^{b/d} BM cells were examined by immunofluorescent analysis (40× magnification). **A** Both CD31 and VE-Cadherin (red) were used as markers to identify the endothelial cells. H-2K^b-positive cells (green) indicate that they are BM-derived cells. The colocalization area (yellow) demonstrate that these endothelial cells are derived from the BM cells. **B** Three markers Desmin, α-SMA and PDGFR-β (red) were used to identify pericytes. H-2K^b positive cells (green) are BM derived cells. The merged images (yellow) reveal that a portion of the pericytes are BM-derived

Fig. 4.

Migration of BM cells into subcutaneous tumors and other organs. **A** Subcutaneous tumors and normal lung, liver and spleen tissues from H-2K^{b/d}-transplanted mice were examined for the presence of H-2K^b cells using immunohistochemical analysis (10× magnification). The colocalization of H-2K^b and CD31 positive cell (yellow, arrow) was shown in the subcutaneous tumor only. **B** Mouse BM cells were labeled with CM-Dil red fluorescent dye and intravenously injected into mice. Mice were killed 1 or 7 days after injection. The *first* and *second panels* show lung, liver, and spleen tissues from control mice without tumors. The *third* and *fourth panels* show lung, liver, and spleen tissues from mice with lung tumors. Lung, liver, and spleen tissues were examined for the presence of CM-Dil-labeled cells using a fluorescent microscope (10× magnification)