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# Role of Macrophage Targeting in the Antitumor Activity of Trabectedin

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## SUMMARY

There is widespread interest in macrophages as a therapeutic target in cancer. Here, we demonstrate that trabectedin, a recently approved chemotherapeutic agent, induces rapid apoptosis exclusively in mononuclear phagocytes. In four mouse tumor models, trabectedin caused selective depletion of monocytes/macrophages in blood, spleens, and tumors, with an associated reduction of angiogenesis. By using trabectedin-resistant tumor cells and myeloid cell transfer or depletion experiments, we demonstrate that cytotoxicity on mononuclear phagocytes is a key component of its antitumor activity. Monocyte depletion, including tumor-associated macrophages, was observed in treated tumor patients. Trabectedin activates caspase-8-dependent apoptosis; selectivity for monocytes versus neutrophils and lymphocytes is due to differential expression of signaling and decoy TRAIL receptors. This unexpected property may be exploited in different therapeutic strategies.

## INTRODUCTION

The last decade witnessed an ever-growing awareness of the promoting role of chronic inflammation in cancer initiation and progression (Balkwill and Mantovani, 2001; Ben-Neriah and Karin, 2011; Coussens and Werb, 2002; Mantovani et al., 2008; Murray and Wynn, 2011). Cancer-related inflammation is now recognized as a hallmark of tumors (Colotta et al., 2009; Hanahan and Weinberg, 2011). Tumor-associated macrophages (TAM) are present in large numbers in tumor tissues and are key promoters of cancer-related inflammation (Allavena and Mantovani, 2012; DeNardo et al., 2009; Joyce and Pollard, 2009; Mantovani et al., 2002; Martinez et al., 2009; Pollard, 2004; Qian and Pollard, 2010).

### Significance

Tumor-associated macrophages (TAM) elicit cancer-promoting inflammation and have been implicated in cancer progression and resistance to therapies, thus representing attractive therapeutic targets. Trabectedin is a recently approved chemotherapeutic agent of marine origin that is clinically active in different tumors. Here, we report that trabectedin selectively depletes in vivo mononuclear phagocytes, including TAM. Evidence is provided that macrophage targeting is a key component of the antitumor activity of trabectedin. Selective mononuclear phagocyte depletion occurs in tumor patients who receive trabectedin-based therapy. These findings shed unexpected light on the mode of action of a clinically useful anticancer agent, provide strong proof-of-concept evidence for macrophage targeting in humans, and open interesting perspectives for the rational exploitation of this peculiar property in therapeutic settings.

Several mouse and human studies have shown that high TAM density is mostly associated with poor patient prognosis and resistance to therapies (DeNardo et al., 2011; Mazzieri et al., 2011; Steidl et al., 2010). Based on these findings and general paradigms, TAM are considered attractive targets for antitumor interventions (Balkwill and Mantovani, 2010; Hanahan and Coussens, 2012). Strategies to exploit TAM as therapeutic target include re-education (Beatty et al., 2011; Duluc et al., 2009; Guiducci et al., 2005; Rolny et al., 2011), deletion by killing or blocking of recruitment (DeNardo et al., 2011; Qian et al., 2011; van Rooijen and van Kesteren-Hendrikx, 2003; Zhang et al., 2010), and modulation by chemotherapeutic agents (Apetoh et al., 2007). Monocyte/macrophage depletion in experimental settings has been successful in limiting tumor growth and metastatic spread and in achieving better responses to conventional chemotherapy and antiangiogenic therapy (Mazzieri et al., 2011; Qian et al., 2011).

Trabectedin (ET-743) is a new DNA binder of marine origin that is approved in Europe and other countries as a single agent for the treatment of soft tissue sarcoma after failure of doxorubicin or ifosfamide and in relapsed platinum-sensitive ovarian cancer patients in combination with pegylated liposomal doxorubicin (Le Cesne et al., 2012; Grosso et al., 2007; Monk et al., 2010). From a mechanistic point of view, trabectedin shows features different from those of other conventional chemotherapeutic agents: it binds the minor groove of DNA and, in addition to blocking the cell cycle, it affects gene transcription and DNA-repair pathways (D'Incalci and Galmarini, 2010; Erba et al., 2001; Minuzzo et al., 2000). Furthermore, differentiation of human liposarcoma cells after therapy was described (Charytonowicz et al., 2012; Forni et al., 2009). We previously reported that trabectedin is selectively cytotoxic in vitro to human monocytes and inhibits the production of some cytokines (e.g., CC chemokine ligand 2 [CCL2], interleukin-6) functionally relevant in the tumor milieu (Allavena et al., 2005; Germano et al., 2010). These studies as well as clinical evidence (D'Incalci and Galmarini, 2010; Grosso et al., 2007) suggested that trabectedin may not only hit neoplastic cells but also affect the tumor microenvironment.

The present investigation was designed to elucidate the role of macrophage targeting in the antitumor activity of this drug in vivo by using four different mouse tumor models. We further studied the effect on mononuclear phagocytes in soft tissue sarcoma patients receiving trabectedin.

## RESULTS

### Trabectedin Selectively Decreases the Number of Mononuclear Phagocytes in Blood and Spleens of Tumor-Bearing Mice

We tested whether trabectedin affects mononuclear phagocytes upon treatment in vivo of tumor-bearing mice. Three transplantable tumor models were used (MN/MCA1 fibrosarcoma, ID8 ovarian carcinoma, and Lewis lung carcinoma [LLC]), as well as primary fibrosarcoma induced by injection of methylcholanthrene (MCA-fibrosarcoma). After each treatment (once per week for three cycles), blood was collected and leukocytes analyzed by flow cytometry. Figures 1A and 1B show representative experiments with MN/MCA1, ID8, and MCA-fibrosarcoma tumors. Data for LLC tumors are shown in Figure S1A (available online). Treatment with trabectedin caused a rapid decrease (24–48 hr) in the number of blood monocytes ($CD45^+$ $CD11b^+$ $CD115^+$ [macrophage colony-stimulating factor receptor]), while neutrophils ($CD45^+$ $CD11b^+$ $CD115^{neg}$ $SSC^{high}$), CD3 T cells, and CD19 B lymphocytes were unaffected (Figure 1A; Figure S1A). Mean inhibition in the percentage of monocytes/$CD45^+$ progressively increased after each treatment: 42%, 52%, and 86% after cycles 1, 2, and 3, respectively (Figure 1C; $p < 0.001$, Student's t test). Polymorphonuclear neutrophils (PMN)/$CD45^+$ were not reduced after the first two cycles but were slightly inhibited (24%) after the third treatment. Next, we analyzed which monocyte subset was mostly affected. In untreated tumor-bearing mice, 71%–89% of $CD115^+$ monocytes were $Ly6C^{high}$, while $Ly6C^{Inter}$ and $Ly6C^{low}$ cells were 7%–13% and 1.7%–22%, respectively. After treatment, only the $Ly6C^{high}$ subset (also defined as "inflammatory") was strongly inhibited and the remaining monocytes had mostly a $Ly6C^{Inter}$/$Ly6C^{low}$ phenotype (Figure 1B).

The fibrosarcoma MN/MCA1 triggered a marked myelopoiesis that usually started at day +16 postinoculum and was significantly reduced by treatment, especially in terms of monocytes (Figure S1B). To rule out that the effect on monocytes was not merely due to a global inhibition of myelopoiesis, trabectedin was administered at day +25, when myelopoiesis was already full blown (total $CD45^+$ cells increased up to 4-fold). A single injection caused, 48 hr later, a drastic decrease in the absolute number of monocytes but not in the number of PMN or $CD45^+$ cells (Figure 1D). These results demonstrate that trabectedin directly affects circulating monocytes and that this rapid effect is not mediated via inhibition on bone marrow (BM) progenitors. Notably, trabectedin did not modulate the expression of relevant hematopoietic growth factors (macrophage colony-stimulating factor [M-CSF], granulocyte colony-stimulating factor, granulocyte-monocyte colony-stimulating factor, interleukin-3, and interleukin-6) in fibrosarcoma of treated mice (Figure S1C).

At the BM level, mature monocytes ($CD11b^+$ $CD115^+$), but not PMN or total CD45 cells, were significantly reduced (Figure S1D). We next checked whether this preferential effect on the monocytic lineage was also reflected on myeloid progenitors in comparison to other leukocyte lineages. After two cycles of treatment, all immature progenitors were reduced (Figure S1E). This effect is likely due to the cell-cycle-blocking activity of the drug on highly proliferating cells and was observed only after the second and third cycle of treatment.

We next checked the effect of trabectedin on tissue macrophages. The percentage of splenic $F4/80^+$ macrophages was significantly decreased after treatment (Figure 2A). Furthermore, among $CD11b^+$ $GR1^+$ cells, the $Ly6C^{high}$ component (monocytes) was reduced while $Ly6C^{low}$ $Ly6G^+$ cells (granulocytes) were not, underlining the peculiar selectivity of trabectedin for the monocytic lineage. To further validate this result, splenocytes from control tumor-bearing mice were treated in vitro with trabectedin. Annexin $V^+$ apoptotic cells were present only in the $Ly6C^{high}$ monocytic component, while $Ly6G^+$cells were not affected (Figure 2B). Because $CD11b^+$ $GR1^+$ $Ly6C^{high}$ cells have been associated with the operationally defined myeloid derived suppressor cells (MDSC) (Movahedi et al., 2008; Sica and Bronte, 2007; Youn et al., 2012), we investigated whether

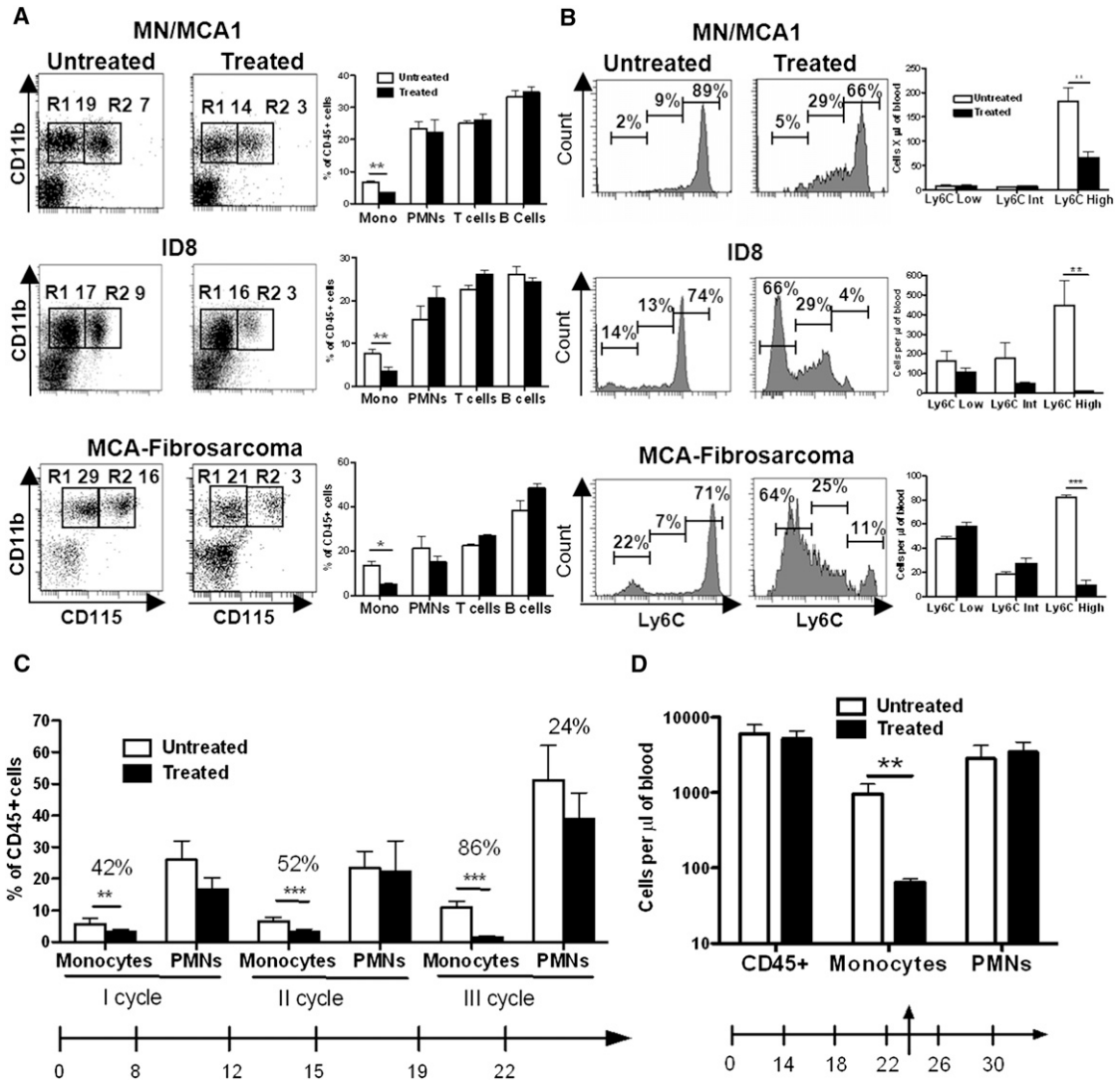


**Figure 1. Trabectedin Decreases Blood Ly6C$^{high}$ Monocytes in Tumor-Bearing Mice**

The transplantable tumors fibrosarcoma MN/MCA1 and the ovarian carcinoma ID8 were intramuscularly inoculated. Primary MCA-fibrosarcomas were induced by intramuscular injection of methylcholanthrene. Trabectedin treatment (0.15 mg/kg) was given intravenously once per week for 3 weeks.

(A) Left: Flow cytometry analysis of blood leukocytes after two cycles of trabectedin (day +16). Monocytes (CD11b$^+$ CD115$^+$, gate R2) are reduced after treatment in all the three tumor models. Right: Percentage of monocytes, PMN (CD11b$^+$ CD115$^{neg}$ SSC$^{high}$), T cells (CD3$^+$), and B cells (CD19$^+$) relative to total CD45$^+$ in control and treated mice (mean ±SD of three experiments with six mice per group for MN/MCA1; mean ±SD of six mice per group for ID8 and MCA-fibrosarcoma). Only monocytes are significantly reduced after treatment.

(B) Left: Representative histograms of blood Ly6C$^{high}$, Ly6C$^{int}$, and Ly6C$^{low}$ monocytes (gated as CD11b$^+$ CD115$^+$ cells) in control and treated tumor-bearing mice. Right: Absolute number per microliter of blood of monocyte Ly6C subsets (mean ±SE of four to six mice per group).

(C) Percentage of monocytes and PMN/CD45$^+$ cells 24 hr after each treatment cycle (mean ±SD of two experiments with six mice per group). Inhibition (%) after treatment is marked on top of the bars.

(D) Trabectedin is directly cytotoxic to blood monocytes. A single injection of trabectedin (day +25), when myelopoiesis is full blown, strongly reduces the number of monocytes. Absolute numbers per microliter of blood (mean ±SD, six mice per group). Total CD45$^+$ cells and PMN are not affected.

Statistical analysis: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t test). See also Figure S1.

trabectedin modulated their immune-suppressive activity. Purified CD11b$^+$ GR1$^+$ Ly6C$^{high}$Ly6G$^{neg}$ splenocytes from tumor-bearing mice were cocultured with lymphocytes from OT-1 mice in the presence of the model antigen ovalbumin. A similar dose-dependent inhibition of lymphocyte proliferation was observed in both control and in vivo-treated mice (Figure 2C). Therefore, trabectedin does not have an impact on the functional activity of MDSC but does significantly reduce the number of Ly6C$^{high}$ cells, the most suppressive subset. Of note, Ly6C$^{low}$ Ly6G$^+$ cells did not have significant suppressive activity (not shown).

## Antitumor Activity of Trabectedin and Selective Depletion of TAM

The antitumor activity of trabectedin on MN/MCA1 fibrosarcoma, lung LLC, and MCA-induced fibrosarcoma is shown in Figures S2A–S2D. Treatment significantly delayed tumor

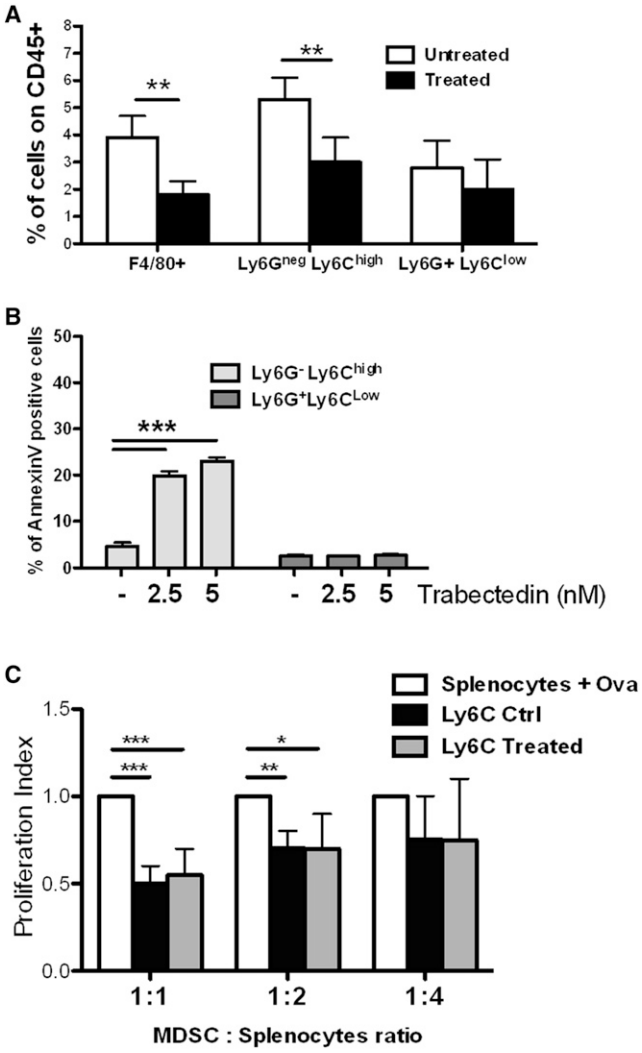


**Figure 2. Effect of Trabectedin on Splenic Myeloid Cells from Tumor-Bearing Mice**

(A) Spleen macrophages ($CD11b^+$ $F4/80^+$) and $CD11b^+Ly6G^{neg}Ly6C^{high}$ myeloid cells are significantly decreased in treated mice bearing the MN/MCA1 fibrosarcoma (24 hr after two cycles of treatment, mean ±SD of three experiments with six mice per group). $CD11b^+$ $Ly6G^+$ $Ly6C^{low}$ cells are not reduced.

(B) Splenocytes of tumor-bearing mice treated in vitro with trabectedin (12 hr). Only $CD11b^+$ $Ly6G^{neg}$ $Ly6C^{high}$ myeloid cells undergo significant apoptosis, evaluated as percentage of Annexin $V^+$ cells. Statistical analysis: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's t test).

(C) Effect of trabectedin on the suppressive activity of spleen MDSC from tumor-bearing mice. Purified $CD11b^+$ $Ly6G^{neg}$ $Ly6C^{high}$ MDSC significantly inhibit the proliferation of splenocytes from OT-1 mice stimulated with ovalbumin. MDSC from mice treated with trabectedin have similar suppressive activity compared with MDSC from untreated mice.

growth; lung metastasis from MN/MCA1 was also significantly reduced (Figure S2B). To assess the leukocyte infiltrate, tumors were excised at the end of treatment and analyzed by flow cytometry. The percentage of TAM ($CD11b^+$ $F4/80^+$ /$CD45^+$) in treated mice was significantly lower in all four tumor models (mean inhibition: MN/MCA1, 38%; LLC, 46%; ID8, 43%; MCA-fibrosarcoma, 30%; Figure 3A). The percentage of T cells and neutrophils was never reduced; T cells actually showed a tendency to increase.

Because tumors of treated mice were significantly smaller in size, we postponed tumor cell inoculum in control mice in order to have treated and untreated tumors of comparable size at the end of the experiment. As shown in Figure 3B, TAM ($CD11b^+$ $F4/80^+$) were strongly reduced after treatment, from 60% to 13%; of note, tumors excised 8 days after the last treatment still had lower TAM, indicating that the effect of trabectedin was long-lasting. The phenotype of TAM comprises mature ($F4/80^+Ly6C^{low}$) and immature ($F4/80^+$ $Ly6C^{int/high}$) macrophages (Movahedi et al., 2010; Swirski et al., 2007). In untreated mice, most TAM had a mature phenotype (80% $Ly6C^{low}$; Figure 3C); in mice receiving trabectedin, up to 75% of TAM had an immature $Ly6C^{high}$ profile. This finding suggests that trabectedin inhibits the local differentiation of tumor-recruited monocytes to fully mature macrophages. TAM are M2-like polarized macrophages (Mantovani et al., 2008; Solinas et al., 2010) with low expression of MHC II molecules ($MHC^{low}$), although a proportion of M1-like $MHC^{high}$ macrophages are present as well (Movahedi et al., 2010). Treatment of MN/MCA1-bearing mice resulted in a similar inhibitory effect on $CD11b^+$ $Ly6C^{int}$ $MHC^{high}$ (TAM/M1-like) and $Ly6C^{int}$ $MHC^{low}$ (TAM/M2-like) (Figure 3D).

Size-comparable tumors were used for immunohistochemistry studies. $CD68^+$ and $Dectin^+$ TAM in treated tumors were reduced by more than 50% (Figure 4A). The production of CCL2, a major determinant of monocyte recruitment in tumors, was also decreased in treated mice (Figure 4B). The predominant CCL2-expressing cells appeared as dispersed macrophages; sorted TAM from in vivo-treated tumors indeed showed significantly lower CCL2 messenger RNA levels (Figure S3A). The angiogenic network of treated tumors was also significantly decreased in terms of microvessel density and maturation, as assessed by the number of CD31 and CD105 (endoglin)-positive cells as well as staining for vascular endothelial growth factor (VEGF) (Figure 4C).

Given the capacity of TAM to promote tumor cell proliferation and metastasis (Joyce and Pollard, 2009; Sica and Mantovani, 2012), we investigated the impact of trabectedin on the functional TAM-tumor cell interaction. In vitro studies with florescence-activated cell-sorted TAM from untreated mice cocultured with MN/MCA1 cells showed that TAM significantly enhanced tumor cell proliferation and their migration through Matrigel-coated transwells (Figures S3B and S3C). When TAM were pretreated with trabectedin (under noncytotoxic conditions: 5 nM, 2 hr), these protumor properties were partially abrogated; of note, the expression of matrix metalloproteinases 9 and 12 by tumor cells was significantly downregulated (Figure S3D).

## Role of TAM Targeting in the Antitumor Activity of Trabectedin

The results discussed above raised the question whether targeting of mononuclear phagocytes plays a major role in the antitumor activity of trabectedin. This was addressed by taking advantage of tumor cell lines that were rendered resistant to its cytotoxic activity upon continuous exposure to increasing drug concentrations. The trabectedin-resistant subline of the MN/MCA1 fibrosarcoma (MN/MCA1-RES) showed stable in vitro

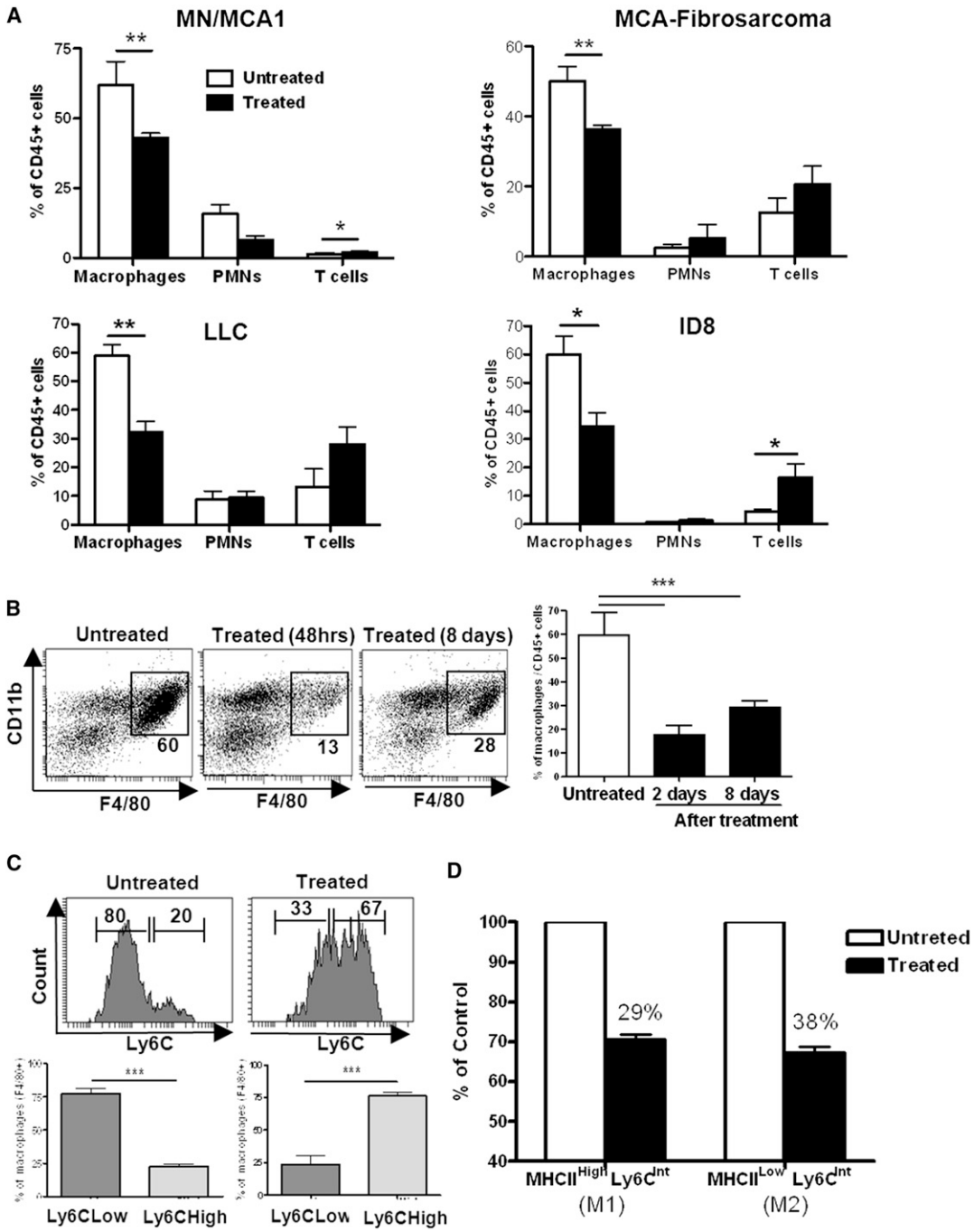


**Figure 3. Trabectedin Decreases Tumor-Associated Macrophages after In Vivo Treatment**

Transplantable tumors (MN/MCA1, LLC, ID8) and MCA-induced fibrosarcoma were excised 48 hr after the third cycle of trabectedin and analyzed in flow cytometry.

(A) Percentage of TAM ($CD45^+$ $CD11b^+$ $F4/80^+$), PMN ($CD45^+$ $CD11b^+$ $Ly6G^+$), and T cells ($CD45^+$ $CD3^+$) relative to total CD45 cells; (mean ±SD with six to eight mice per group; for MN/MCA1, mean ±SD of three experiments).

(B) Dot plots of TAM ($CD45^+$ $CD11b^+$ $F4/80^+$) in treated and untreated size-comparable tumors. Tumor cell inoculum in untreated mice was postponed to have tumors of comparable size at the end of treatment (volume-untreated tumors: 2,900 ± 537 $mm^3$; volume-treated tumors: 3,220 ± 407 $mm^3$). Bars depict the percentage of $CD11b^+$ $F4/80^+$ on $CD45^+$ live cells (mean ± SD, six mice/group).

(C) Representative flow cytometry of Ly6C expression on $F4/80^+$ TAM. In trabectedin-treated tumors, TAM contain more $Ly6C^{high}$ macrophages. Data are expressed as percentage of $F4/80^+$ cells (mean ±SD of two experiments with six mice per group).

(D) Susceptibility of TAM subsets to trabectedin. TAM ($CD45^+$ $CD11b^+$ $Ly6C^{Int}$) from untreated or treated tumors were analyzed for MHC II expression. TAM MHC $II^{High}$ (M1) and MHC $II^{Low}$ (M2) were equally susceptible to trabectedin.

Statistical analysis: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Student's t test). See also Figure S2.

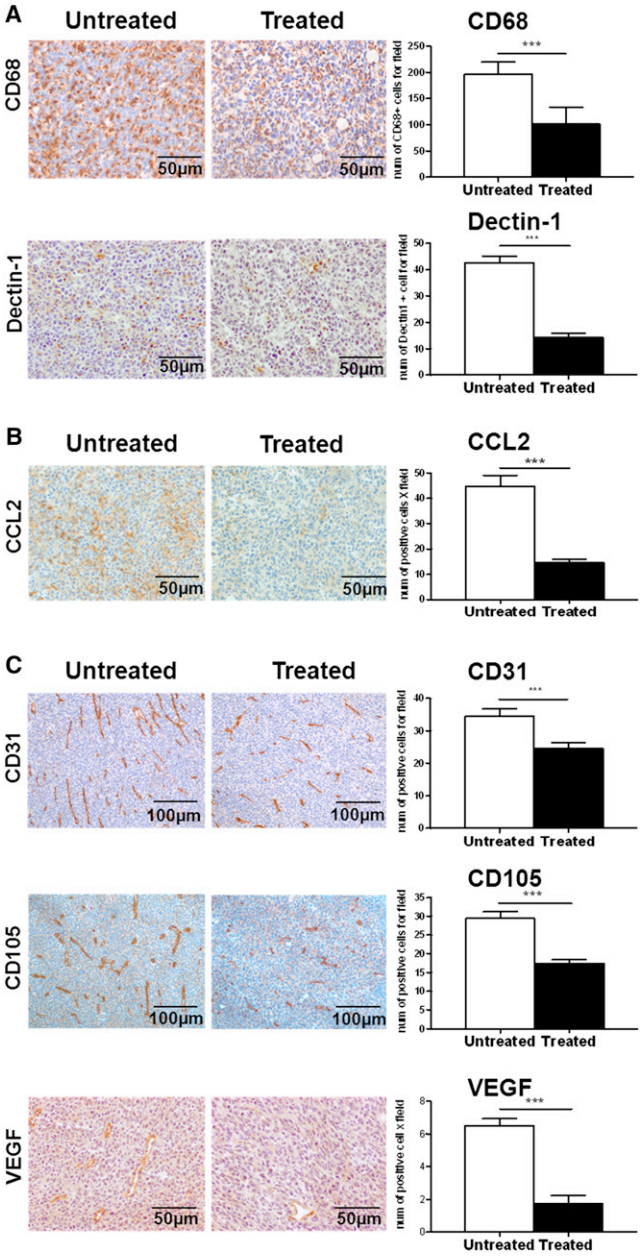


**Figure 4. Trabectedin Decreases TAM, the Chemokine CCL2, and Angiogenesis in Treated Tumors**

Immunohistochemistry was performed on size-comparable MN/MCA1 tumors (see legend to Figure 3) after three cycles of treatment. Results are shown as representative pictures (20×) and as mean values ±SD number of positive cells per five microscope fields for each sample, with six mice per group.

(A) Staining of intratumor macrophages with anti-CD68 monoclonal antibody (mAb) and anti-Dectin-1 mAb.

(B) Staining of tumor sections with anti-CCL2 mAb.

(C) Staining of intratumor vessels with CD31, CD105 (endoglin) and VEGF.

Statistical analysis: $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's t test). See also Figure S3.

and ex vivo resistance (>8-fold) after growth in mice (Figure 5A; Figure S4A). Of note, trabectedin displayed strong in vivo antitumor activity on MN/MCA1-RES tumors (Figure 5A) similar to that on wild-type MN/MCA1 cells (shown in Figure S2A). TAM density was also significantly decreased in MN/MCA1-RES tumors after treatment (Figure 5A). Comparable results were found in a xenograft model of ovarian cancer (IGROV) where an IGROV-RES subline, 5-fold resistant to trabectedin (Figure S4B), was at least as responsive to treatment in vivo as the wild-type one (Figure S4C). These results suggested that the antitumor efficacy of trabectedin in drug-resistant tumors could be mediated via its effects on the tumor microenvironment and associated myelomonocytic cells.

To further test this hypothesis, we adoptively transferred myeloid cells after each drug treatment; transfer of BM-derived in vitro-differentiated macrophages from tumor-bearing untreated mice significantly reinstated tumor growth in treated mice (Figure 5B). A similar finding was obtained when BM-derived monocytes were injected, although less marked than with already differentiated macrophages (Figure 5C). In contrast, the transfer of splenic MDSC had no significant effect (Figure 5D). In an effort to further explore the role of macrophage depletion in the antitumor activity of trabectedin, we compared its effects with the macrophage-depleting agent clodronate. Mice bearing MN/MCA1-RES tumors were treated with clodronate liposomes, alone or in combination with the drug. Clodronate single treatment delayed tumor growth initially (Figure 5E); however, when therapy was suspended (day +21), these tumors grew faster than those treated with trabectedin. Combined treatment with clodronate and trabectedin did not result in additive or synergistic antitumor activity. Clodronate liposomes reduced TAM density to a similar extent as trabectedin, but the latter caused a more persistent macrophage depletion (data not shown), possibly as a consequence of inhibition of CCL2 production. Overall, these results are consistent with the view that targeting of TAM is, at least in part, responsible for the antitumor activity of trabectedin under these conditions.

## Selective Depletion of Monocytes and TAM in Tumor Patients Treated with Trabectedin

To assess the actual clinical relevance of the above observations, we investigated the impact of trabectedin on blood monocytes of soft tissue sarcoma patients receiving trabectedin as a single treatment.

Figure 6A shows a representative flow cytometry analysis from a leiomyosarcoma patient whose monocytes decreased after therapy from 9.4% to 3.2%, as well as the absolute number of monocytes but not that of granulocytes and lymphocytes (Figure 6B). Human $CD14^{+}$ $CD16^{low}$ monocytes are considered the counterpart of mouse $CCR2^{+}$ $Ly6C^{high}$ cells (Geissmann et al., 2010); this population was monitored throughout subsequent cycles of therapy and significant reduction after each cycle was seen in two different patients ($p = 0.0109$ for pre- versus post-therapy levels; Figure 6C). We also investigated by immunohistochemistry whether trabectedin caused a decrease of human TAM density. In selected soft tissue sarcoma patients receiving the drug as neoadjuvant treatment, we had access to tumor biopsy specimens before therapy and to post-therapy surgical samples. A strong decrease in the density of TAM and blood vessels was evident after treatment (Figure 6D), confirming that trabectedin-mediated depletion of mononuclear phagocytes also occurs in tumor patients.

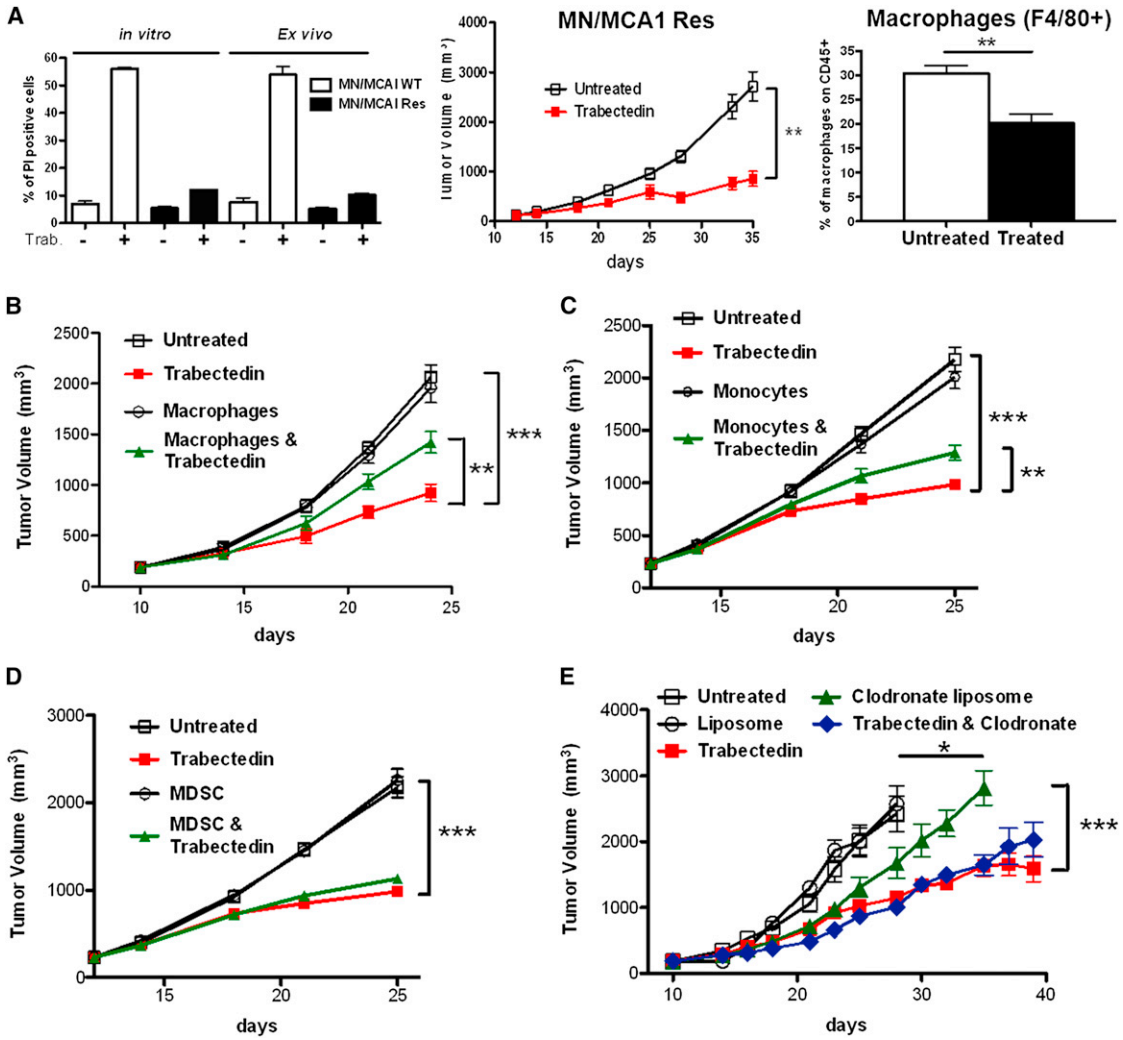


**Figure 5. Role of Macrophage Targeting in the Antitumor Activity of Trabectedin**

(A) Left: In vitro and ex vivo resistance to trabectedin of MN/MCA1-RES cells explanted from tumor-bearing mice and treated in vitro with trabectedin (5 nM). Results are percentage of propidium iodide-positive cells. Middle: Trabectedin has antitumor activity on the drug-resistant variant MN/MCA1-RES grown in mice. Right: Percentage of F4/80$^+$ TAM is reduced in MN/MCA1-RES-bearing mice treated with trabectedin (mean ±SD of six mice per group).

(B) Adoptive transfer of BM-derived in vitro M-CSF-differentiated macrophages into mice bearing MN/MCA1-RES tumors reinstates tumor growth. Macrophages ($2 \times 10^6$/mouse) were intravenously injected 72 hr after each of the three cycles of trabectedin (tumor volume, mean ±SD of six mice per group).

(C) Adoptive transfer of BM-purified monocytes into mice bearing MN/MCA1-RES tumors reinstates tumor growth. Monocytes were intravenously injected ($2 \times 10^6$/mouse) 72 hr after each treatment (tumor volume, mean ±SD of six mice per group).

(D) Adoptive transfer of purified spleen MDSC into mice bearing MN/MCA1-RES tumors does not reinstate tumor growth. MDSC were intravenously injected ($2 \times 10^6$/mouse) 72 hr after each treatment (tumor volume, mean ±SD of 6 mice per group).

(E) Comparison of trabectedin treatment with clodronate liposomes. Mice bearing MN/MCA1-RES tumors were treated with trabectedin, clodronate liposomes, or their combination. The effect of trabectedin is similar to clodronate but lasts longer (tumor volume, mean ±SD of six to eight mice per group).

Statistical analysis: *$p < 0.05$, ***$p < 0.001$ (Student's t test). See also Figure S4.

## Mechanism of Macrophage Depletion by Trabectedin

In an effort to elucidate the peculiar selectivity of trabectedin for the myelomonocytic lineage, we investigated in depth the pathways of apoptotic death induced by the drug in different human leukocyte subsets. Mitochondrial depolarization and release of cytochrome C in the cytosol were observed only in treated monocytes, while PMN and T cells were unaffected (Figure 7A). Furthermore, trabectedin caused the cleavage of caspase-8 and caspase-9 selectively in monocytes (Figure 7B). In a kinetic analysis, activation of caspase-8 occurred earlier than caspase-9 or mitochondria depolarization (Figures S5A and S5B) and Z-IETD-FMK (a caspase-8 inhibitor) strongly reduced Annexin V$^+$ cells (Figure S5C). Thus, the first temporal event induced by trabectedin in monocytes is the activation of caspase-8. This caspase is the key effector molecule of the extrinsic apoptotic pathway mediated by membrane death receptors (Fas and tumor necrosis factor-related apoptosis inducing ligand [TRAIL] receptors [TRAIL-Rs]) (Bodmer et al., 2000). Expression of TRAIL and Fas receptors by purified human monocytes, neutrophils, and T lymphocytes is shown in Figure 7C (mean ± SE, n = 6

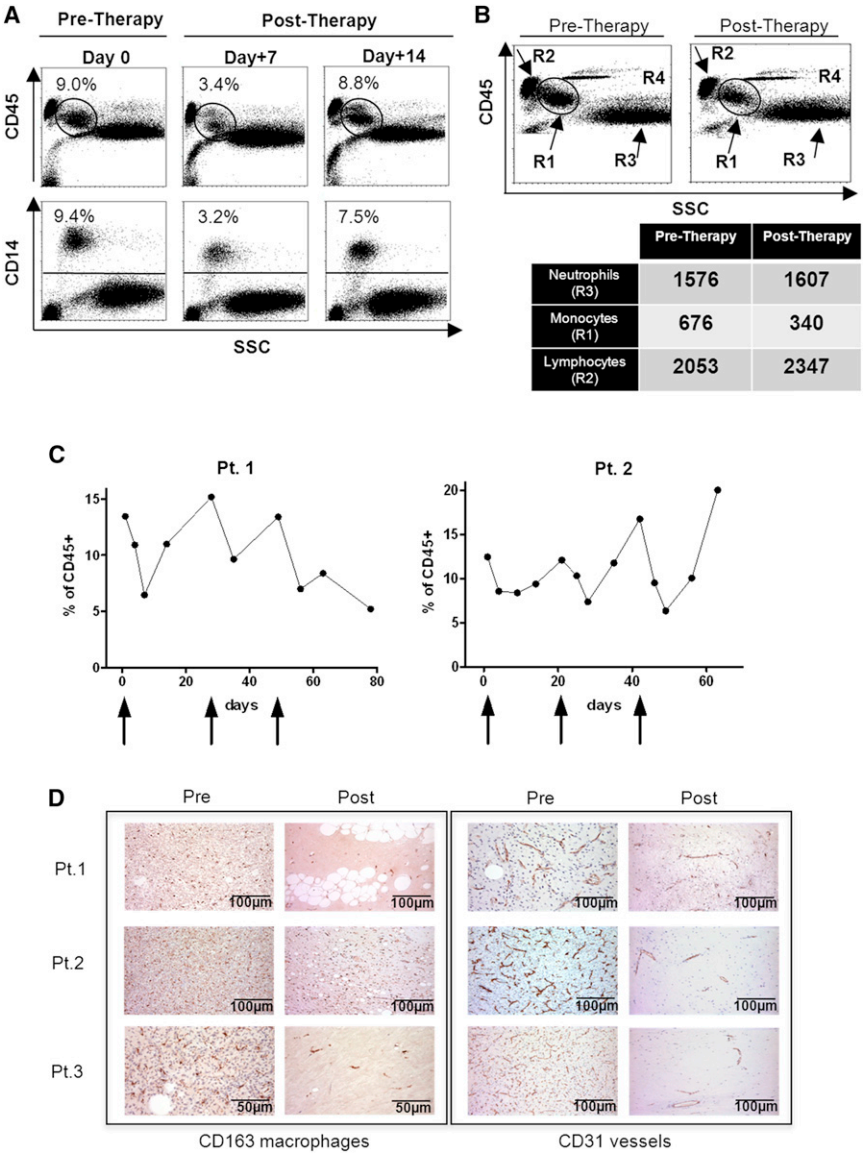


**Figure 6. Trabectedin Decreases Blood Monocytes in Soft Tissue Sarcoma Patients**

(A) Representative dot plots of peripheral blood samples stained for CD45 and CD14 from a leiomyosarcoma patient after trabectedin therapy. Blood was collected the day before therapy and 7 and 14 days postinfusion.

(B) Representative dot blots of peripheral blood samples before and after therapy; CD45 versus side scatter (SSC) dot plots showing decrease in the number of CD14 monocytes (circular gate).

(C) Reduction in the number of blood monocytes in two soft tissue sarcoma patients treated with trabectedin over different therapy cycles, indicated by the arrows. Results expressed as percentage $CD14^+$ $CD16^-/CD45^+$.

(D) Immunohistochemistry of tumor sections from three different soft tissue sarcoma (STS) patients receiving trabectedin therapy prior to surgery. Sections marked (PRE) are bioptic samples before treatment. Sections marked (POST) are surgical samples after treatment. Tumor macrophages (CD163) and vessels (CD31) are reduced after treatment.

experiments). Fas receptor was very high and equally expressed in all three subsets. TRAIL receptor 1 (TNFRSF10A or R1) and especially TRAIL receptor 2 (TNFRSF10B or R2) were expressed in monocytes and low or absent in T cells and neutrophils. In contrast, the decoy nonsignaling TRAIL receptor 3 (TNFRSF10C or R3) was highly expressed by neutrophils and T cells but very low in monocytes. A second decoy receptor (TNFRSF10D or R4) was not expressed by any subset (not shown). Therefore, the expression pattern of TRAIL-Rs strikingly parallels the susceptibility of leukocytes to the cytotoxic action of trabectedin, with monocytes being susceptible and neutrophils and lymphocytes being resistant.

Next, the contribution of death receptors to trabectedin-induced apoptosis was investigated. We first set up a flow cytometry technique to specifically detect cleaved caspase-8 in leukocytes. Figure S5D shows a typical experiment where trabectedin dose- and time-dependently activates caspase-8 in monocytes but not in neutrophils and T cells. Cell pretreatment with blocking anti-R1 and anti-R2 (alone or in combination) strongly inhibited caspase-8 activation (Figure 7D). As reasonably expected, anti-R3 (decoy R) antibodies had no inhibitory effect. When used on neutrophils, pretreatment with anti-R3 did not result in higher susceptibility (not shown), in line with their lack of signaling R1 and R2 receptors. Anti-Fas antibodies had no significant effect. In parallel experiments, we verified that recombinant TRAIL ligand induced caspase-8 activation in monocytes and that anti-TRAIL-Rs antibodies properly blocked this activation (Figure S5E). These results indicated that caspase-8 activation via TRAIL-Rs was responsible for monocyte apoptosis induced by trabectedin. To further confirm our interpretation, we overexpressed the decoy R3 receptor, fused with green florescent protein, in the myeloid cell line HL60 and in 293T cells. In R3-expressing cells (Figure S5F), activation of caspase-8 was strongly reduced compared to mock-transfected cells, either with trabectedin and TRAIL ligand (Figure 7E; Figure S5G), confirming the involvement of TRAIL receptors in trabectedin-induced apoptosis.

We next investigated TRAIL-R expression in mouse leukocytes. In mice, only one signaling receptor is present (TRAIL-R2 or DR5). Blood monocytes with the phenotype $Ly6C^{high}$ (which are more susceptible to the cytotoxic effect of trabectedin, see Figure 1B) expressed higher TRAIL-R2 compared to $Ly6C^{low}$ monocytes and, in line with the human data, neutrophils and T cells had very low or no expression of TRAIL-R2 (Figure 8A). Activation of caspase-8 by trabectedin was confirmed in mouse macrophages differentiated in vitro from BM cells (Figure 8B). Upon in vivo treatment with trabectedin, TRAIL-$R2^+$

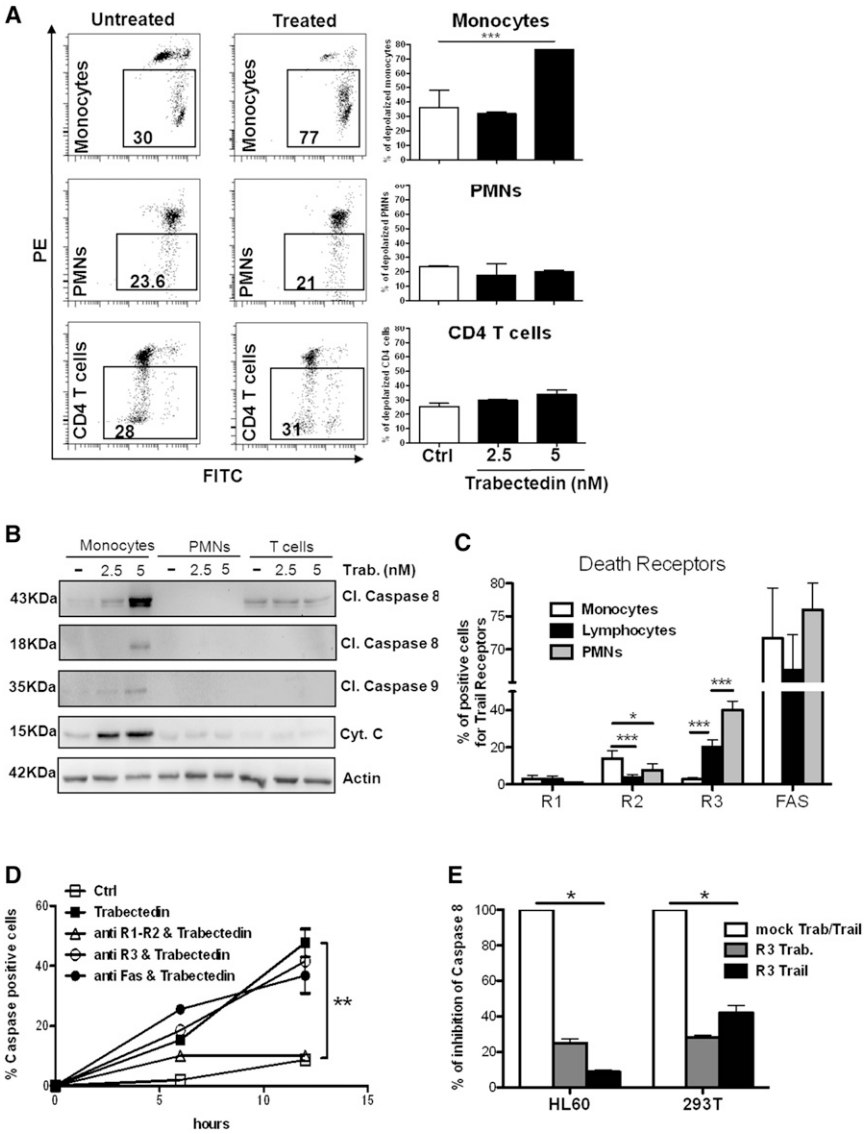


**Figure 7. Mechanism of Apoptosis and Selective Effect of Trabectedin on Monocytes**

(A) Mitochondrial membrane depolarization in human purified monocytes, PMN, and T cells treated with trabectedin (5 nM) for 12 hr.

(B) Western blot analysis of cleaved caspase-8, caspase-9, and cytochrome C in human monocytes, PMN, and T cells treated with trabectedin for 24 hr.

(C) Expression of TRAIL receptors in leukocyte subsets by flow cytometry. Monocytes express the functional TRAIL receptor (R2) and lack the nonsignaling decoy receptor R3. Neutrophils and T cells express high levels of R3 and low or no R1 and R2. Mean ± SD of six different experiments.

(D) Caspase-8 activation induced by trabectedin in purified monocytes is inhibited by anti-TRAIL-R1/2 antibodies. Anti-R3 or anti-Fas antibodies had no inhibitory effect. Results are percentage of cells with cleaved caspase-8 evaluated by flow cytometry.

(E) Overexpression of TRAIL-R3 strongly reduces the activation of caspase-8 in cells treated with trabectedin (10 nM, gray bars) and in cells treated with recombinant TRAIL ligand (500 ng/ml, black bars). Results are expressed as percentage of mock-transfected cells.

Statistical analysis: $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's t test). See also Figure S5.

blood monocytes and TRAIL-R2$^{+}$ TAM in tumors were dramatically reduced (Figure 8C). This result was confirmed also by immunohistochemistry: the density of TRAIL-R2$^{+}$ tumor-infiltrating cells was significantly lower in treated mice (Figures 8D and 8E).

We conclude that trabectedin activates the extrinsic apoptotic pathway downstream of TRAIL receptors and that selectivity for mononuclear phagocytes versus neutrophils and lymphocytes is due to differential expression of signaling and decoy receptors.

## DISCUSSION

Trabectedin is a recently approved drug of marine origin that is particularly active in soft tissue sarcoma and ovarian cancer (Carter and Keam, 2010; Grosso et al., 2007; Monk et al., 2010). Substantial clinical and experimental evidence suggests that this antitumor agent may importantly modulate the tumor microenvironment (D'Incalci and Galmarini, 2010; Germano et al., 2010; Grosso et al., 2007). In this study, we demonstrate that trabectedin rapidly activates caspase-8, the key effector molecule of the extrinsic apoptotic pathway, in mononuclear phagocytes. This effect is remarkably selective for myelomonocytic cells, as neutrophils and lymphocytes are not affected. In four different mouse tumor models, including two lines representative of its clinical spectrum of action, and in a chemically induced fibrosarcoma model, trabectedin caused significant and selective reduction of blood monocytes, in particular of the Ly6C$^{high}$ phenotype and of spleen and tumor macrophages. We also demonstrate that TAM targeting is a key determinant of its efficacy, as trabectedin retained antitumor activity in vivo against sarcoma and ovarian carcinoma cells rendered resistant to its cytotoxic action. This was demonstrated by cell transfer of monocytes and macrophages, after drug infusion, that re-established tumor growth in the fibrosarcoma model, supporting the concept that at least part of its antitumor activity is mediated via targeting of protumoral macrophages. Furthermore, the immune suppressive monocytic subset (CD11b$^{+}$ Ly6C$^{high}$) of MDSC was significantly reduced by trabectedin.

The remarkable susceptibility of the myelomonocytic lineage was ascribed to a differential expression of signaling and decoy TRAIL receptors among leukocytes: monocytes express functional receptors and lack the decoy R3, whereas neutrophils and T cells predominantly express R3. Involvement of TRAIL-Rs

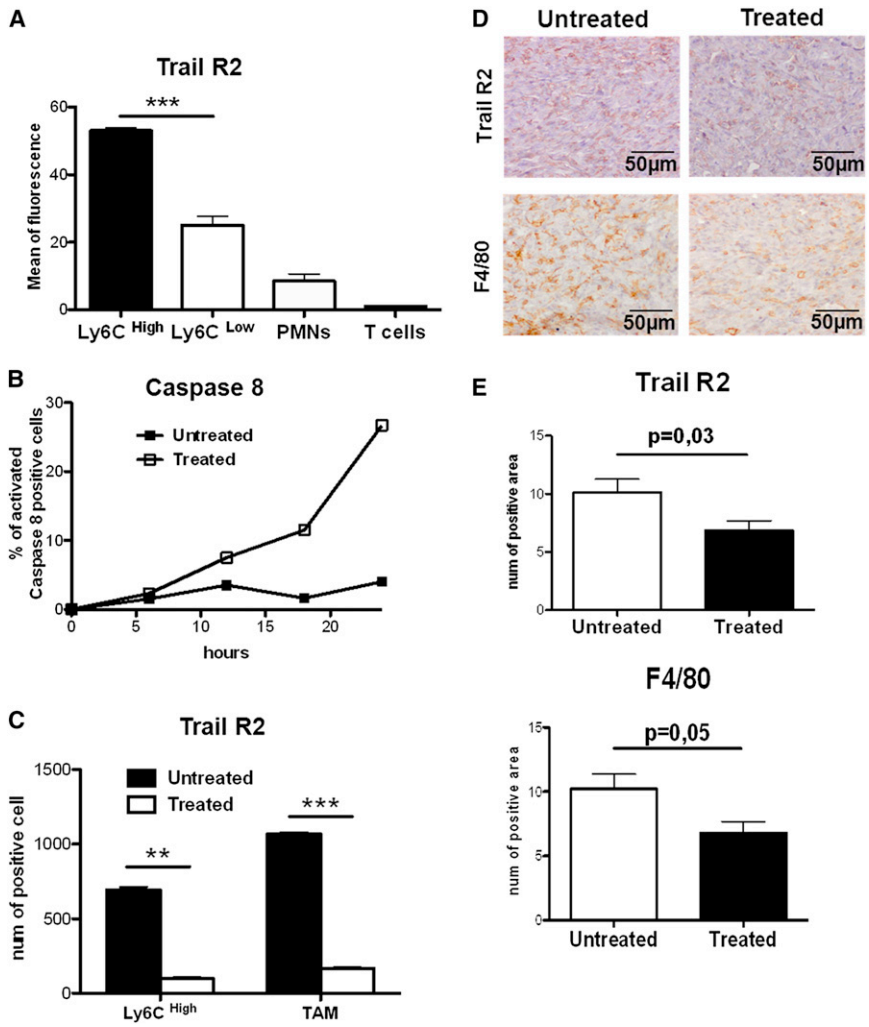


**Figure 8. Mouse Blood Monocytes and TAM Expressing TRAIL-R2 Decrease in Trabectedin-Treated Mice**

(A) Blood Ly6C$^{high}$ monocytes express higher levels of TRAIL-R2; neutrophils and T cells have low or no expression, as evaluated by flow cytometry.

(B) Kinetic of caspase-8 activation induced by trabectedin (10 nM) in mouse macrophages differentiated in vitro from BM cells). Results are % of cells with cleaved caspase-8 evaluated by flow cytometry.

(C) In vivo treatment with trabectedin strongly reduces the number of blood Ly6C$^{+}$ TRAIL-R2$^{+}$ monocytes and F4/80$^{+}$ TRAIL-R2$^{+}$ TAM (mean ±SD of four mice per group).

(D) Immunohistochemistry of MN/MCA1-RES tumors from untreated and treated mice stained with anti-TRAIL-R2 and anti-F4/80.

(E) The statistical analysis was performed on four different tumors for each group (mean of a total of 15 microscope fields).

Statistical analysis: **p < 0.01, ***p < 0.001 (Student's t test).

in trabectedin-induced apoptosis was demonstrated by reduced caspase-8 activation in the presence of anti-TRAIL-Rs antibodies and because overexpression of the decoy R3 resulted in marked inhibition of cleaved caspase-8 in trabectedin-treated cells. Furthermore, we demonstrate that mouse Ly6C$^{high}$ monocytes and TAM express TRAIL-R2 and in vivo treatment with trabectedin strongly decreases the number of TRAIL-R2$^{+}$ myelomonocytic cells both in blood and tumors.

Although it was previously claimed that normal cells are resistant to TRAIL, under pathological conditions, such as viral or bacterial infections, leukocytes can be killed, as shown for HIV-infected T cells and alveolar macrophages (Benedict and Ware, 2012; Herbeuval et al., 2005; Steinwede et al., 2012). The intriguing question is why mononuclear phagocytes express functional death receptors in homeostatic conditions and, unlike other leukocytes, are not protected by the presence of the decoy receptor. Activated macrophages can be very reactive cells and have a long life span in tissues, whereas neutrophils survive for just few days. It may be possible, therefore, that the presence of TRAIL-R on mononuclear cells is a checkpoint for controlling their overreactivity. Indeed, TRAIL-R$^{-/-}$ mice have exaggerated innate immune responses with increased levels of interleukin-12, interferon-α and interferon-γ (Diehl et al., 2004). We noticed that TRAIL-R2 is not expressed by all TAM within tumors. Further studies will clarify the nature of these TRAIL-R2$^{+}$ macrophages. Of note, in human macrophages differentiated in vitro, both M1- and M2-polarized cells expressed TRAIL-Rs (not shown).

The precise mechanism by which trabectedin initiates caspase-8 cleavage remains to be elucidated. It is unlikely that this compound directly engages TRAIL-Rs on cell membrane (based on modeling experiments, not shown). Caspase-8 activation generally starts with the oligomerization of death receptors in response to ligand binding; however, there is evidence that receptor activation may occur independent of the cognate ligand. This ligand-independent activation has been described for several members of the tumor necrosis factor receptor family, including TRAIL-Rs (Cazanave et al., 2011; Chan et al., 2000; Clancy et al., 2005; Lim et al., 2011), and was mechanistically explained by the overexpression of death receptors and/or by their recruitment into lipid rafts on cell membrane. Receptor modulation has been widely studied using pharmacological or chemical/physical agents (Shirley et al., 2011; Siegelin, 2012). Interestingly, some active compounds that are able to upregulate TRAIL-Rs are derived from natural products: animal venoms and bacterial toxins (Park et al., 2012). In origin, trabectedin is also a natural product derived from a marine organism. In an effort to investigate its mode of action, a transcriptional profiling was conducted in mononuclear phagocytes, and key relevant data confirmed at the transcript and protein level. A short treatment (6 hr) upregulated *TRAIL-R2* and *FADD* (Fas-associated protein with death domain), an important proapoptotic gene, while *TRAIL* ligand was not modulated (Figure S5H). Enhanced levels of TRAIL-R2

and FADD, induced by trabectedin, may indeed facilitate the recruitment of caspase-8 and the activation of the apoptotic cascade.

Effects other than macrophage depletion may account for the antitumor efficacy of trabectedin. We show that in treated murine tumors the vessel network and the chemokine CCL2 were significantly downmodulated. TAM depend on the recruitment of monocytes from the blood compartment, mediated by signals including chemokines; although in situ proliferation has been recently identified as a determinant of macrophage accumulation at sites of M2-polarized inflammation (Jenkins et al., 2011) and can be sustained in tumors by paracrine circuits based on M-CSF and its receptor (Bottazzi et al., 1990), the role of CCL2 in tumors is of major importance (Bottazzi et al., 1983; Qian et al., 2011). In the fibrosarcoma model, CCL2 is produced mainly by TAM and its expression was dramatically reduced after treatment. Therefore, in addition to inducing direct cytotoxic activity on mononuclear phagocytes, trabectedin could downmodulate the recruitment of circulating monocytes into tumors.

It is well established that TAM drive angiogenesis and this represents a pathway of escape and resistance to anti-VEGF therapy (Ferrara, 2010; Mazzieri et al., 2011; Murdoch et al., 2008; Noonan et al., 2008). The reduced angiogenic network in treated tumors could be the result of an indirect effect due to the decrease in TAM numbers; however, in tumors treated with clodronate liposomes, where TAM were significantly reduced, the number of $CD31^+$ vessels was not decreased (data not shown). VEGF was also downmodulated in trabectedin-treated tumors; therefore, both direct and indirect effects are likely to account for the reduced angiogenesis. As for the susceptibility of endothelial cells to the drug, we have evidence that they are relatively resistant in vitro (not shown). Interestingly, it was recently reported that tumor endothelial cells may be susceptible to TRAIL killing in a mouse model (Wilson et al., 2012).

In soft tissue sarcoma patients treated with trabectedin-based chemotherapy, a decrease of blood monocytes was observed. We retrospectively collected data from 34 patients from whom laboratory test results and clinical outcome were available, and found that 19 patients (56%) experienced monocyte reduction (range, 30%–77%). These results raised the question whether monocyte depletion correlates with drug responsiveness in treated patients. In a retrospective analysis, and in heavily pretreated patients, this was particularly difficult to address; a trend toward a correlation between decreased monocytes and response to therapy was indeed observed, but the association did not reach statistical significance ($p = 0.078$). Further analysis is needed to elucidate this issue. In addition to blood monocytes, in selected cases we had access to tumor biopsy specimens from patients receiving trabectedin as neoadjuvant therapy. When tumor biopsy specimens (before treatment) were compared to surgically explanted tumors (after treatment), a dramatic reduction of vessels and macrophages was observed. Although the drug may directly affect neoplastic cells in these patients, these results suggest that trabectedin may impact the tumor microenvironment, as observed in mouse tumor models.

In conclusion, we have reported that a clinically useful antitumor agent selectively depletes mononuclear phagocytes in blood and tumor tissues and that macrophage targeting is a key component of its antitumor activity. These findings provide proof-of-concept evidence for the value of macrophage targeting in anticancer therapies in humans and shed unexpected light on the mode of action of an available anticancer agent. Moreover, these observations unveil a different perspective for the exploitation of trabectedin in cancer, for instance, in combination with antiangiogenic therapies or as a tool to limit myelomonocytic cell-mediated immune suppression.

## EXPERIMENTAL PROCEDURES

### Drug

Trabectedin (PharmaMar, Colmenar Viejo, Madrid, Spain) was dissolved in dimethylsulfoxide to 1 mM and kept at −20°C.

### Mice, Tumors, and Primary Cells

Mice were used in compliance with national (4D.L.N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 86/609, OJ L 358, 1, 12-12-1987; *NIH Guide for the Care and Use of Laboratory Animals*, US National Research Council, 1996). This investigation was approved by the Animal Care and Use Committee of the Humanitas Clinical and Research Center.

C57/BL/6J mice were from Charles River (Calco, Como Italy). The transplantable MN/MCA1 mouse fibrosarcoma, the trabectedin-resistant variant MN/MCA1-RES, and the LLC were inoculated intramuscularly ($10^5$ cells). The mouse ovarian carcinoma ID8 (kindly provided by Prof. Balkwill, London, UK) was inoculated intraperitoneally ($10^7$ cells). The human ovarian carcinoma IGROV-1 and the trabectedin-resistant variant IGROV-1-RES were inoculated intramuscularly ($10^6$ cells) in nude mice. Primary fibrosarcomas were induced in C57/BL/6J mice by subcutaneous inoculation of 50 μg methylcholanthrene in 0.2 ml peanut oil (Sigma-Aldrich). Tumors growing progressively over 3 weeks were used. Treatment with trabectedin (0.15 mg/kg/body weight) was administered intravenously once per week for 3 weeks and started when tumors were palpable. For further details, please refer to Supplemental Experimental Procedures.

### Phenotype Analysis

Blood cells were collected from the eye vein of anesthetized mice and splenocytes from disaggregated spleen and filtered through Falcon strainers. Mouse tumors were cut into small pieces, disaggregated with collagenase (0.5 mg/ml), and filtered through strainers. Cells ($10^6$) were stained with specific antibodies and with live and dead dye (Invitrogen, Life Technology) (see Supplemental Experimental Procedures for an antibody list). Flow cytometry was performed by FACS Canto instrument and FACS Diva software version 6.1.1 (BD Biosciences).

### Adoptive Transfer and Depletion of Myeloid Cells

Mouse macrophages were in vitro differentiated from BM cells cultured with 50 ng/ml murine M-CSF for 5 days as described elsewhere (Saccani et al., 2006). Monocytes were isolated from BM using CD115 MicroBead Kit (Miltenyi Biotec, Ausburn, CA). $GR1^+$ cells were isolated from spleen of MN/MCA1-bearing mice with a MDSC isolation kit (Miltenyi Biotech). Cells ($2 \times 10^6$) were transferred intravenously 72 hr after each drug treatment for 3 weeks. Macrophage depletion was performed by clodronate liposome (200 μl) intraperitoneally three times per week for 3 weeks. Control mice received liposome alone (van Rooijen and van Kesteren-Hendrikx, 2003). $GR1^+$ cells were depleted using anti-Gr1 antibody (RB6-8C5, eBioscience, San Diego, CA) and control mice received the isotype-matched LTF-2 antibody (BioXcell, West Lebanon, NH). Antibodies (200 μg/mouse) were injected intraperitoneally three times a week for 3 weeks.

### Apoptosis and Caspase Activation

Human monocytes, neutrophils, and T lymphocytes from blood of healthy donors were purified through density gradients as described previously (Allavena et al., 2005). Cells were treated with trabectedin (2.5–10 nM) or recombinant TRAIL (500 ng/ml; Enzo Lifesciences) for different time points and

apoptosis evaluated as caspase activation and mitochondrial depolarization. Cleavage of caspases was detected by western blotting with anti-cleaved caspase-8 and 9 (Germano et al., 2010). In flow cytometry, cleavage of caspase-8 was detected on permeabilized cells. Pretreatment with blocking antibodies used anti-human TRAIL-R1, TRAIL-R2 (Enzo Lifesciences, Farmingdale, NY), TRAIL R3 (R&D Systems, Minneapolis, MN), anti-human Fas (Millipore, Billerica, MA) at 1 μg/ml. Mouse monocytes were treated with 10 nM trabectedin and stained with anti-mouse cleaved caspase 8 (Cell Signaling). Measurement of mitochondrial potential was performed with the Mito Probe JC-1 assay kit (Invitrogen, Life Technology, Carlsbad, CA) for 30 min at 37°C.

### Human Patients

Soft tissue sarcoma patients received trabectedin therapy as single treatment at Fondazione IRCCS Istituto Nazionale Tumori, Milan. The study was approved by the Institutional Ethical Board and blood samples were obtained upon written informed consent. Patients received multiple cycles of therapy at the dose of 1.3 mg/m$^2$ every 3 weeks as 24 hr infusion (Casali et al., 2010). Leukocytes were collected immediately before and at different days after drug administration. Cells were stained with anti-CD45-FITC, anti-CD14-PerCp, and CD16-PE (BD Biosciences) and analyzed on a FACSCalibur (BD Biosciences) cytofluorimeter. Analysis (1–2 × 106 events) was done by WinMDI 2.9 software.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.01.008.

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