Inhibition of tumor-associated macrophages by trabectedin improves the antitumor adaptive immunity in response to anti-PD-1 therapy

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A considerable proportion of cancer patients are resistant or only partially responsive to immune checkpoint blockade immunotherapy. Tumor-Associated Macrophages (TAMs) infiltrating the tumor stroma suppress the adaptive immune responses and, hence, promote tumor immune evasion. Depletion of TAMs or modulation of their protumoral functions is actively pursued, with the purpose of relieving this state of immunesuppression. We previously reported that trabectedin, a registered antitumor compound, selectively reduces monocytes and TAMs in treated tumors. However, its putative effects on the adaptive immunity are still unclear. In this study, we investigated whether treatment of tumor-bearing mice with trabectedin modulates the presence and functional activity of T-lymphocytes. In treated tumors, there was a significant upregulation of T cell-associated genes, including CD3, CD8, perforin, granzyme B, and IFN-responsive genes (MX1, CXCL10, and PD-1), indicating that T lymphocytes were activated after treatment. Notably, the mRNA levels of the Pdcd1 gene, coding for PD-1, were strongly increased. Using a fibrosarcoma model poorly responsive to PD-1-immunotherapy, treatment with trabectedin prior to anti-PD-1 resulted in improved antitumor efficacy. In conclusion, pretreatment with trabectedin enhances the therapeutic response to checkpoint inhibitor-based immunotherapy. These findings provide a good rational for the combination of trabectedin with immunotherapy regimens.

Keywords: anti-PD-1 · immunotherapy · trabectedin · tumor microenvironment

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

The recent introduction of immunotherapy based on checkpoint inhibitors has shown that it is possible to successfully reactivate the immune system of cancer patients. The proportion of nonresponding patients, however, is still high and this may be due to several causes: intrinsic factors in the tumor, for example, lack of immunogenic antigens; mutations in molecules or signaling

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pathways that are crucial for antigen presentation, checkpoints other than PD-1 and CTLA-4, targeted by the currently available antibodies [1]. Other extrinsic factors involve immune cells and comprise a paucity of CD8+ cytotoxic T cells or the presence of immune cells with suppressive capacity. Among the latter, a predominant role is played by suppressive myeloid cells and macrophages infiltrating the tumor stroma [2, 3].

Tumor-associated macrophages (TAMs) are frequently the most numerous component among myeloid cells of the innate immunity. It is overall established that in advanced tumors, under the influence of products derived from cancer cells, TAMs acquire an immunosuppressive phenotype that hampers an effective antitumor response. TAMs further support cancer proliferation, angiogenesis, and distant spreading, fueling tumor progression [4–11].

Therefore, there is a strong rational to counteract the protumoral and immunosuppressive functions of TAMs. Among the different strategies tested in the last years to target TAMs, one of the most successful has been the inhibition of the CSF1 receptor, specifically expressed on macrophages [12, 13].

Other approaches, such as the use of chemoattractant inhibitors or of bisphosphonates, have been investigated with limited clinical results [4, 10, 14].

Among drugs specifically affecting the survival of myeloid cells, the compound trabectedin holds a special place. Trabectedin is a tetrahydroisoquinoline alkaloid that was originally extracted from a marine organism, the Tunicate Ecteinascidia turbinata, and is now synthetically produced by PharmaMar (Spain) [15–17]. Trabectedin, a registered antitumor agent, is used in the clinic for the second-line treatment of soft tissue sarcoma, especially liposarcoma, and for relapsed platinum-sensitive ovarian cancer patients, in combination with liposomal doxorubicin [18–22]. The mechanism of action of trabectedin on cancer cells is complex and different from that of other anticancer agents. By binding to the minor groove, trabectedin directly interferes with activated transcription to poison the transcription-coupled nucleotide excision repair system and generates double-strand DNA breaks. Further studies demonstrated that it mediates the displacement of oncogenic transcription factors from their target promoters, thereby affecting oncogenic signaling addiction [23, 24].

Our group previously reported that trabectedin has a direct cytotoxic effect on TAMs. Remarkably, this effect is highly specific for monocytic lineage cells, as neutrophils or T lymphocytes are not affected by its cytotoxic action [25]. We further clarified that trabectedin rapidly triggers, within few hours, the activation of caspase 8, via increased expression and aggregation of TRAIL receptors [26, 27]. The differential expression of TRAIL receptors, abundantly expressed only in monocytes, explained the exquisite selectivity of trabectedin for this lineage. Furthermore, at noncytotoxic concentrations, trabectedin inhibits the production of specific inflammatory mediators such as CCL2, IL-6, and CXCL8. This effect was particularly observed in monocytes, TAMs, and in cancer cells such as myxoid liposarcoma and ovarian cancer tumors [25, 26, 28].

Overall, our findings demonstrated that the antitumor activity of trabectedin also involves a specific modulation of the tumor microenvironment, in particular: reduced numbers of infiltrating TAMs; inhibition of monocyte recruitment; inhibited production of cytokines whose activity is important for tumor progression. Our studies also showed that in tumors treated with trabectedin there was a relative increase in T-lymphocytes [26], but it is still unclear about the overall impact of trabectedin on the adaptive antitumor immune response. In this study, we investigated whether trabectedin modulates T-lymphocytes in mouse cancer models and whether trabectedin treatment can improve the therapeutic response to checkpoint inhibitor-based immunotherapy.

Results

The antitumor efficacy of trabectedin treatment is reduced in immunodeficient mice

To evaluate the role of the adaptive immune system in the antitumor efficacy of trabectedin, we used nude mice, lacking T-lymphocytes, and immunocompetent (IC) C57BL/6 mice, in parallel experiments. The animals were inoculated i.m. with the fibrosarcoma MN/MCA1 (5 × 10^4 cells). Trabectedin treatment started as soon as the tumor was palpable. The animals received two doses of trabectedin (0.1 mg/kg), 7 days apart. Treatment with trabectedin reduced tumor growth in both nude (Fig. 1A, red line) and IC mice (Fig. 1B, red line), but treatment efficacy was slightly greater in the latter. In IC mice, trabectedin caused 50% reduction of tumor growth (best T/C 51% on day 21) whereas its effect was lower in nude mice, 33% reduction (best T/C 67% on day 21). These results suggested that the antitumor activity of trabectedin may include the involvement of a host adaptive immune response.

Immunomodulatory effects of trabectedin on peripheral and tumor-infiltrating leukocytes

We had previously reported that trabectedin is selectively cytotoxic for monocytes/macrophages [26]. We checked in the present experiments whether myeloid cells in the circulating blood and in the tumor stroma were affected upon treatment with trabectedin. By analyzing with flow cytometry, the leukocyte composition of blood and tumors in IC mice (gating strategies described in Supporting information Figs. S1,2,4), we confirmed that treatment with trabectedin selectively reduced the number of Ly6C<sup>high</sup> monocytes in the blood and of Ly6C<sup>low</sup> macrophages in tumors (Fig. 2A, D and Supporting information Fig. S3). Ly6C<sup>high</sup> monocytes are the main subset among circulating monocytes, while this marker is lost upon maturation to macrophages; in fact, the Ly6C<sup>low</sup> subset is the predominant one among TAMs, and was significantly reduced after treatment. On the other hand, we did not observe any significant change in neutrophils or DCs (Fig. 2C and F). As for lymphocytes, treatment with trabectedin increased in the tumor mass the relative percentage of CD3+ and CD8+...
Figure 1. Tumor growth of fibrosarcoma tumors in nude mice and in immunocompetent mice upon treatment with trabectedin. The fibrosarcoma MN/MCA1 was inoculated intramuscle at $5 \times 10^4$ cells in: (A) nude mice; (B) immunocompetent (IC) C57BL/6 mice. Trabectedin treatment (red lines) was administered (0.1 mg/kg) as indicated by arrows. Results are expressed as mean volume ± SEM and are representative of two independent experiments (each one with four of five mice per group); nude mice n = 9 per group; C57BL/6 n = 10 per group. Statistical analysis: **p < 0.01, ****p < 0.0001 (unpaired t-test with Welch’s correction).

T lymphocytes (Fig. 2E and Supporting information Fig. S5); in the blood we noticed a slight increase in the percentage of regulatory T cells (Fig. 2C). Overall, the flow cytometry analysis confirmed our previous results that trabectedin selectively reduces the number of monocytes and macrophages but not that of neutrophils and DCs [26].

We further analyzed the mRNA expression of markers and biological mediators of T-lymphocytes, by real-time PCR, in tumors...
of mice treated with trabectedin. As observed in the flow cytometry analysis, the genes coding for CD3 and CD8 were significantly upregulated in treated tumors (Fig. 3A and C), while CD4 and FOXP3 did not show a significant variation (Fig. 3B and D). The genes coding for the serine protease granzyme B and the pore-forming protein perforin, two cytotoxic molecules produced by CD8+ T lymphocytes, were also more expressed (Fig. 3E and F). mRNA levels of IFN-γ in treated tumors were higher (p = 0.06) (Fig. 3G); furthermore, genes activated in response to interferon, such as MX1 and CXCL10, were significantly more expressed (Fig. 3H and I). Of note, the Pdcd1 gene coding for PD-1 and that of its ligand PD-L1 were significantly upregulated (Fig. 3J and K).

To confirm these findings, we performed immunohistochemistry of CD8 and PD-1 markers in tumors derived from mice treated or not with trabectedin. We observed that cytotoxic CD8+ lymphocytes were significantly increased in trabectedin-treated tumors (Fig. 4A and B) and PD-1 staining was detected in tumors derived from both experimental groups, with a prevalence in trabectedin-treated tumors, but this difference did not reach the significant threshold (Fig. 4C and D). Taken together, our results indicate that treatment with trabectedin affects the tumor immune environment leading to an activation of the adaptive immune response against tumors.

The therapeutic response to anti-PD-1 immunotherapy is increased by trabectedin pretreatment

The increased expression of PD-1, IFN-responsive genes, and increased presence of CD8 T cells in trabectedin-treated tumors prompted us to study the combination of trabectedin with anti-PD-1. Schematic protocol is shown in Fig. 5A. We first investigated whether our fibrosarcoma model is responsive to anti-PD-1 monotherapy. Mice were treated with anti-PD-1 or with an irrelevant mAb (0.25 mg/mouse, total of three injections). We did not observe any significant changes in tumor growth with anti-PD-1 alone (Fig. 5B, red line) or with the irrelevant mAb (black line) compared to control tumors (green line). Since therapy with anti-PD-1 not only unlocks the brake imposed by the immune checkpoint but also allows the proliferative expansion of T lymphocytes, we reasoned that a combined synchronous treatment could have
Figure 4. Increased expression of CD8 and PD-1 in tumors treated with trabectedin. Immunohistochemistry of CD8 (A, B) and PD-1 (C, D) were performed on fibrosarcoma tumors treated or not with trabectedin. (A and C) Each dot corresponds to a ROI (region-of-interest) in a tumor slice. Data shown are representative of two independent experiments. Statistical analysis: *p < 0.05 (unpaired t-test with Welch’s Correction). (B and D) Images are representative of two experiments. Magnification 20×.

a deleterious effect of trabectedin on proliferating lymphocytes. Therefore, to evaluate the antitumor efficacy of the combination, we followed a protocol where animals were first treated with trabectedin (days 10 and 17) and subsequently with anti-PD-1 (days 20 to 27) (Fig. 5A). This sequential combination gave a significantly greater therapeutic response (Fig. 5C, red line) compared to the combination of trabectedin + the irrelevant mAb (blue line). Of note, treatment with trabectedin was not affected by the combination with the irrelevant mAb tumors (Fig. 5D).

We next checked by immunohistochemistry the immune landscape of tumors treated with the combination protocol, and we observed an increased infiltration of CD8+ lymphocytes. Figure 6 shows that CD8-positive area was higher in tumors treated with trabectedin + anti-PD-1 compared to trabectedin or anti-PD-1 monotherapy. All these data indicate that treatment with trabectedin is able to enhance anti-PD-1 immunotherapy, also in tumors otherwise resistant to checkpoint inhibitors.

Discussion

Trabectedin has multiple mechanisms of action on cancer cells; in addition, an increasing number of publications report that its antitumor activity is related also to its ability to modulate the tumor microenvironment. We described immunomodulatory effects on innate immunity cells, in particular, an apoptotic-inducing effect on monocytes/macrophages and the inhibition of some inflammatory cytokines and chemokines. The macrophage-depleting ability of trabectedin has been confirmed by other groups [29–35]. In a mouse model of orthotopic pancreatic cancer, trabectedin specifically reduced the number of circulating monocytes, while PMNs were not significantly affected [29]. Similar findings were observed in an orthotopic mouse model of osteosarcoma, in melanoma, Ewing sarcoma, and in skeletal metastasis of prostate cancer [30–33]. In hematological malignancies, trabectedin not only had cytotoxic effects on neoplastic cells but also induced the apoptotic death of associated myeloid cells [35–37].

In this study, we focused our attention on adaptive immunity cells, in particular, cytotoxic CD8+ T cells, which are the ultimate effectors against tumors. We observed that in trabectedin-treated tumors there is an upregulation of cytotoxic molecules, such as granzyme B and perforin, and an increased expression of IFN-γ, PD-1, and of IFN-responsive genes (MX1 and CXCL10), overall suggesting an activation of the antitumor T cell-mediated immune response. Furthermore, an increased number of T cells were detected in treated tumors. This result may be in part explained by the relative decrease of macrophages induced by trabectedin; however, it cannot be excluded that the upregulation of
Figure 5. The therapeutic response to anti-PD-1 immunotherapy is increased by pretreatment with trabectedin. (A) Sequential treatment schedule of trabectedin followed by anti-PD-1 mAb. (B) Tumor growth curve of MN-MCA1 fibrosarcoma in untreated mice (Ctrl), mice treated with anti-PD-1 mAb, or with irrelevant mAb (Irrelev). (C) Tumor growth curve in mice treated with the combination of trabectedin + irrelevant mAb (red line) or the combination of trabectedin + anti-PD-1 (red line). (D) Tumor growth curve in mice treated with trabectedin alone (Trab), or with Trab + irrelevant mAb. Data are pooled from two independent experiments and expressed as mean ± SEM of tumor volume (each experiment with seven-ten mice in the control or anti-PD-1 groups; five-seven mice in the trabectedin ± irrelevant or ± anti-PD-1 groups). Statistical analysis: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (two-way Anova). Statistical significance refers to tumors treated with the combination of trabectedin + irrelevant mAb versus irrelevant mAb alone (blue and black lines), and to tumors treated with the combination of trabectedin + anti-PD-1 versus trabectedin + irrelevant mAb (red and blue lines).

the chemokine CXCL10 has contributed to recruit new T lymphocytes within the tumor mass.

These findings are in line with those of a previous study by Borgoni et al. in collaboration with our group, in mouse pancreatic cancer [29]. The TME of orthotopically growing pancreatic tumors is highly immunosuppressive. The authors found that treatment with trabectedin alleviated the local immunosuppression; in particular, they reported that tumor-infiltrated lymphocytes sorted from treated tumor showed a switch from an IL10-secreting phenotype toward an effector phenotype, with an increased percentage of IFN-γ+ T cells. Furthermore, a sharp increase in the percentage of Eomes+ and PD-1+ T cells, consistent with a cell memory population, was observed in CD8+ T cells.

These data indicate an important immunomodulatory effect of trabectedin on the adaptive immunity leading to an antitumor phenotype (IL10 low/IFN-γ high).

Of interest, Borgoni et al. suggested that trabectedin may also have direct effects on lymphocytes. They reported that when CD4+ T cells were stimulated with supernatants from macrophages, previously exposed in vitro to trabectedin, there were epigenetic modulations at the Il10 promoter, indicative of a negative regulation of IL10 production and, by contrast, an increased activation of the T-bet promoter. Furthermore, a negative modulation of the Il10 promoter was noted also in CD4 T cells directly exposed to trabectedin in vitro. These results indicate that possible direct effects of trabectedin on T cells cannot be excluded [29].

The question whether the promising results obtained with checkpoints inhibitors can be further improved by combination with anticancer drugs, is clinically relevant. Certainly, research to find out the most suitable combination is crucial, also considering that most chemotherapeutic agents cause immunosuppression and, thus, they might antagonize the effects of immunotherapies. The results shown in the present article suggest that trabectedin’s mode of action renders it an ideal candidate for these combinations. Several laboratories—including ours—have deeply investigated the complexity of the mode of action of trabectedin, that involves a direct effect on cancer cells mediated by its ability to cause DNA damage and modify transcription regulation, and an indirect effect due to its ability to modulate the tumor microenvironment and immune response [15, 16, 26]. These latter properties make trabectedin a suitable molecule to be combined with anti-PD-1 antibodies. In fact, although the preclinical models used in our studies were only marginally sensitive to
anti-PD-1 antibodies, the combination with trabectedin was more efficient, with a clear evidence of activated adaptive responses against the tumor and increase in PD-1 expression. Thus, these preclinical studies provide a rational to test the combination of trabectedin and anti-PD-1 antibodies in the clinic.

Materials and methods

Mice

Mice were used in compliance with national (D.L. N. 26, G.U. 4, March 2014) and international law and policies (EEC Council Directive 2010/63/EU, OJ L 276/33, September 22, 2010; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 2011). Experimental protocols have been reviewed and approved by the IRFMN Animal Care and Use Committee that includes members “ad hoc” for ethical issues, and by the Italian Ministry of Health. Nude and C57BL/6 8-week-old male mice were purchased from Charles River (Calco, Milano). Tumor cells $5 \times 10^4$ from the fibrosarcoma MN-MCA1 were injected i.m. Tumor volume was measured with a caliper.

Treatments

Trabectedin (PharmaMar, Spain), 0.1 mg/kg was administered i.v. when tumors were first palpable: at days 13 and 19 (IC mice), or at days 10 and 17 (nude mice) post-tumor cell injection. Trabectedin was reconstituted in water for injection and further diluted in saline immediately before use. Monoclonal antibody anti-PD-1 (BioXcell; clone RMPI-14; catalogue number BE0146) or anti-irrelevant antibody (BioXcell, clone 2A3; catalogue number BP0089 suggested by BioXcell company) were injected at 1 mg/kg i.p. The antitumor activity of treatments was verified as tumor volume measured by caliper and calculated as tumor growth inhibition: T/C ratio (treated/control $\times 100$).

Flow cytometry

The flow cytometry experiments were performed following “Guidelines for the use of flow cytometry and cell sorting in immunological studies” [39], using the following mAbs:

Bulk leukocyte analysis CD45 PerCP (BD Biosciences, Eysins, Switzerland; clone 30-F11; catalogue number 557235); Cd11b Pacific Blue (Biolegend, San Diego, CA, USA; clone M1/70; catalogue number 101224); Cd11b PE (eBioscience, San Diego, CA, USA; clone 2A3; catalogue number BP0089 suggested by BioXcell company) were injected at 1 mg/kg i.p. The antitumor activity of treatments was verified as tumor volume measured by caliper and calculated as tumor growth inhibition: T/C ratio (treated/control $\times 100$).

Figure 6. Infiltration of CD8+ T lymphocytes in tumors is increased upon sequential treatment of trabectedin + anti-PD-1. (A) Immunohistochemistry analysis (CD8 positive area) performed on treated fibrosarcoma tumors receiving the indicated treatments. Each dot corresponds to a ROI (region-of-interest) in a tumor slice. A sum of 13–15 ROIs were analyzed for each tumor. Data are representative of two independent experiments. (B) Representative images of CD8 positive staining in tumor sections. Statistical analysis: $**p < 0.01$, $***p < 0.001$ (unpaired t-test with Welch's Correction).
DC analysis CD45 PerCP, CD19 FITC, CD8 PE-Cy7 (BD biosciences; clone 53–6.7; catalogue number: 552877) and CD11c PE (BD Biosciences; clone HL3; catalogue number: 553802); CD11b Pacific Blue (Biolegend); SIGLEC H eFluor 660 (ebioscience; clone eBio440c; catalogue number: 50-0333-82).

T-cell subpopulation analysis CD45 PerCP, CD3 PeCy7 and CD8 Alexa Fluor 647 (BD Biosciences; clone 53–6.7; catalogue number: 557682); CD11b Pacific Blue (Biolegend); CD4 FITC (Biolegend; clone RM4-5; catalogue number: 100510), Foxp3 PE (BD Biosciences; clone MF23; catalogue number: 560408), and CD25 PE-Cy7 (BD Biosciences; clone 53–6.7; catalogue number: 552877). Reactions were developed with 3,3′-diaminobenzidine, DAB (Biocare Medical), then counterstained with hematoxylin and mounted with Eukitt. The analysis was performed with Image Pro software on pictures at the same magnification.

Statistical analysis was performed using unpaired Student's t-test with Welch's correction or two-way Anova. p-value less than 0.05 was considered significant.

Real-time RT-PCR
PureZOL RNA isolation reagent (Bio-Rad) was used to perform total RNA extraction. cDNA was synthesized by random priming from 2 μg of total RNA with GeneAmp RNA PCR kit (Applied Biosystems), following the manufacturer's instructions. Real-time PCR was performed using SYBR Green dye and 7900HT Fast Real-time PCR Systems (Applied Biosystems). For each gene, the sequences of specific primer pairs (Sigma) were designed with Primer Express Software (Applied Biosystems) (Table 1).

mRNA was normalized to GAPDH as:

\[
\Delta CT = CT (a \text{ target gene}) - CT (GAPDH)
\]

Then, we calculated the fold increase using the following formula:

\[
2^{-\Delta \Delta CT}, \text{where} \quad \Delta \Delta CT = \Delta CT (\text{trabectedin sample}) - \Delta CT (\text{average of control sample})
\]

Immunohistochemistry
Tissues were collected, frozen in OCT (Fig. 6) or paraffin embedded (Fig. 4 and Supporting information Fig. S3). Frozen tissues were cut at 10 μm and fixed with fresh PFA 4% for 15 min. Endogenous peroxidases were blocked with 0.03% H2O2 for 5 min and then rodent block M was used to block unspecific binding sites. Sections were incubated with Biotin Rat Anti-Mouse CD8 (Invivogen, cat. n.14-0081-82) for 1 h in humid chamber. Paraffin-embedded murine tissues were cut at 3 μm and placed on superfrost slides. After dewaxing and rehydration, antigen unmasking was performed with Decloaking Chamber in DIVA.
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**Abbreviations:** IC: immunocompetent - TAMs: tumor-associated macrophages; MX1, myxoma resistance protein 1 - MX1: myxoma resistance protein 1