




## Research Article

# 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 immunosuppression. 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, pre-treatment with trabectedin enhances the therapeutic response to checkpoint inhibitor-based immunotherapy. These findings provide a good rationale 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<sup>+</sup> 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 anti-tumor response. TAMs further support cancer proliferation, angiogenesis, and distant spreading, fueling tumor progression [4–11].

Therefore, there is a strong rationale 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 monocytes and macrophages, 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 non-cytotoxic 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.

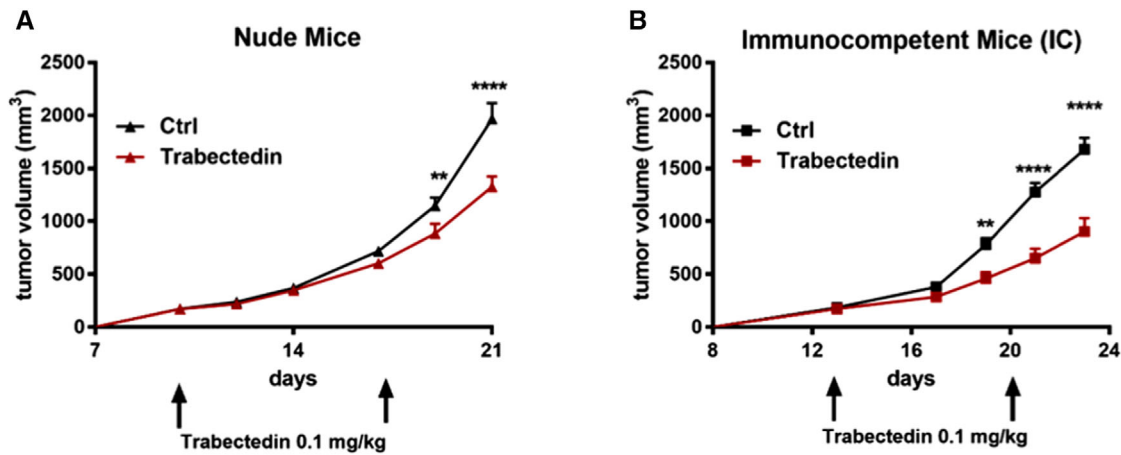
## Results

### 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 ( $5 \times 10^4$  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<sup>+</sup> and CD8<sup>+</sup>

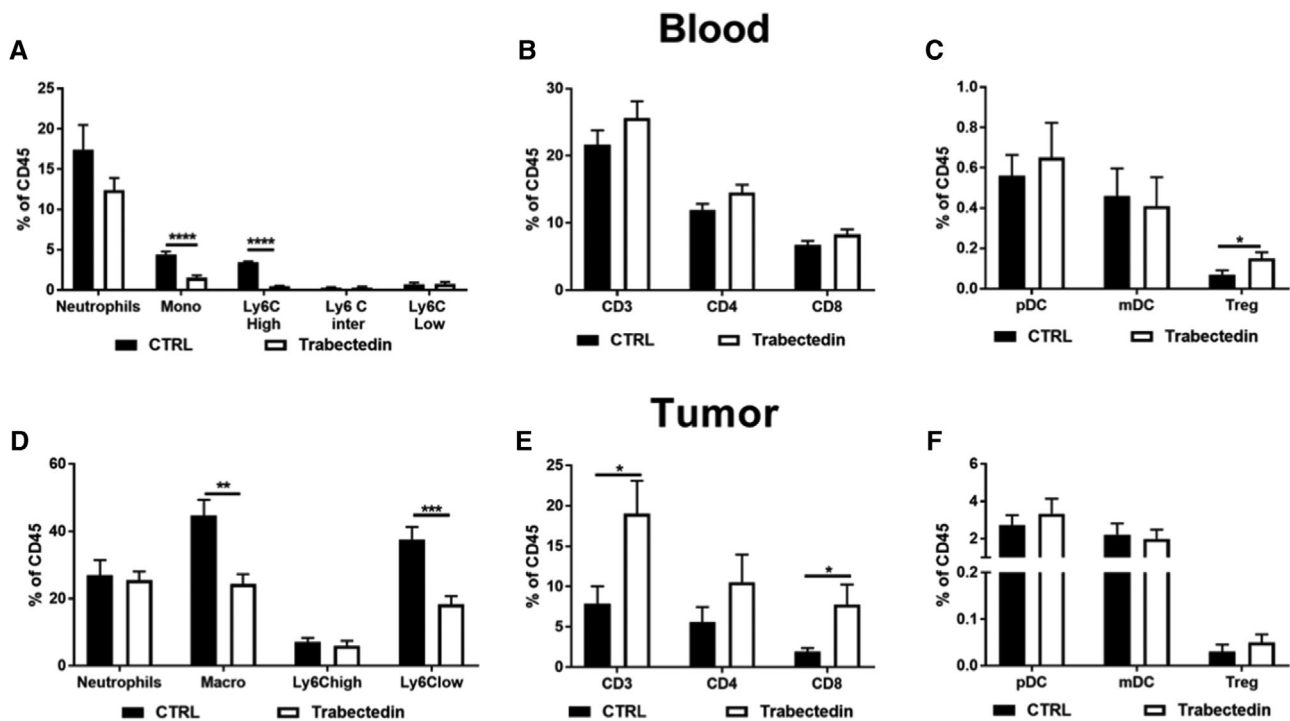


**Figure 1.** Tumor growth of fibrosarcoma tumors in nude mice and in immunocompetent mice upon treatment with trabectedin. The fibrosarcoma MN/MCA1 was inoculated intramuscle at  $5 \times 10^4$  cells in: (A) nude mice; (B) immunocompetent (IC) C57BL/6 mice. Trabectedin treatment (red lines) was administered (0.1 mg/kg) as indicated by arrows. Results are expressed as mean volume  $\pm$  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).

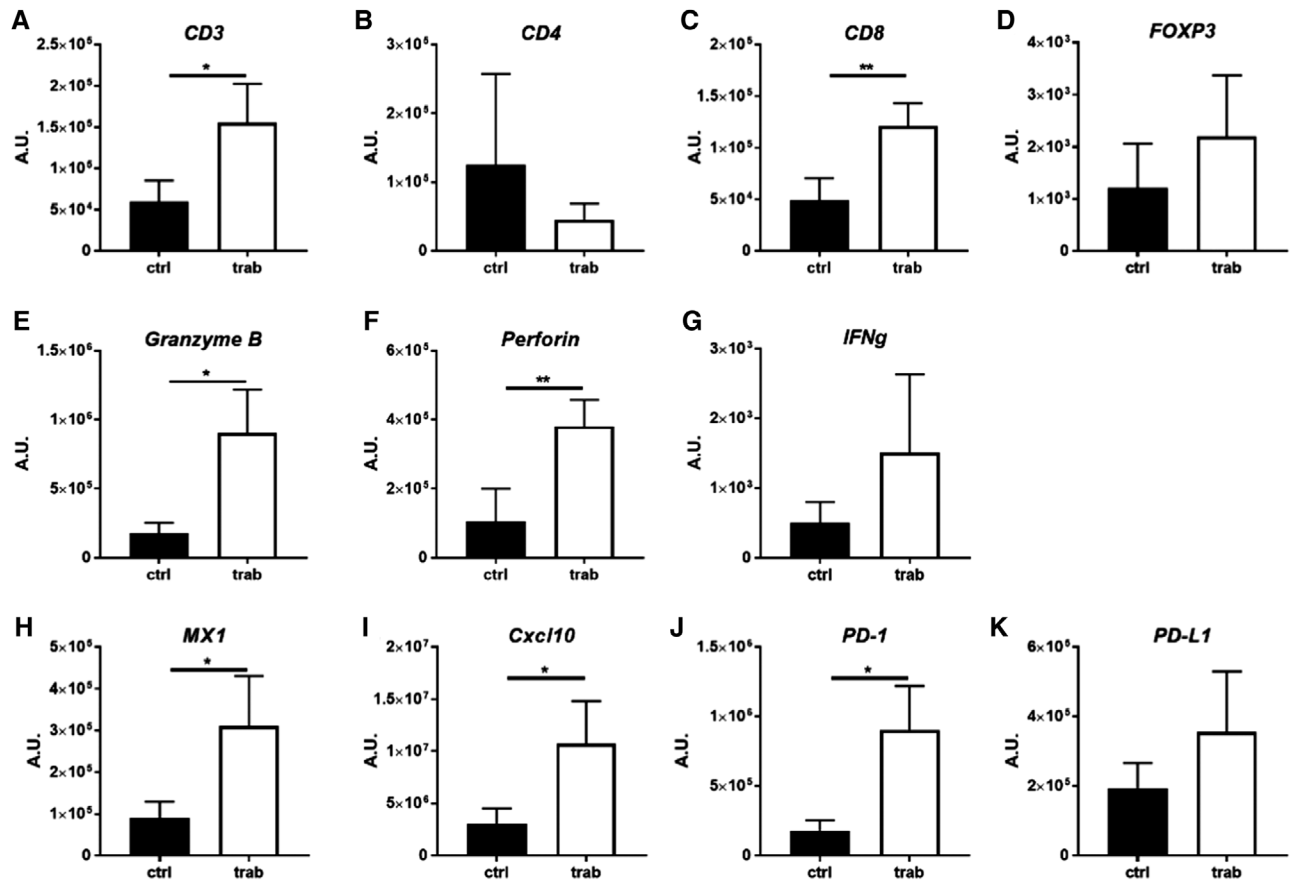
T lymphocytes (Fig. 2E and Supporting information Fig. S5); in the blood we noticed a slight increase in the percentage of regulatory T cells (Fig. 2C). Overall, the flow cytometry analysis confirmed our previous results that trabectedin selectively reduces

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



**Figure 2.** Flow cytometry analysis of blood and tumor leukocytes in tumor-bearing mice treated with trabectedin. Blood (A–C) and tumor-infiltrating (D–F) leukocyte composition in mice treated with trabectedin. The cells were labeled with three different antibodies mix to dissect myeloid, lymphoid, and DC compartment. The analysis was performed compared to the total CD45+ leukocytes. Monocytes and macrophages are reduced by trabectedin treatment, while neutrophils and dendritic cells are not. Lymphocytes (CD3+ and CD8+) are increased in tumors. Results are expressed as mean  $\pm$  SEM of two independent experiments (five mice/group). Statistical analysis: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  (unpaired t-test with Welch's correction). Abbreviations: PMN: neutrophils; Mono: monocytes; pDC: plasmacytoid dendritic cells; mDC: myeloid dendritic cells; Treg: T-regulatory cells; Macro: macrophages.



**Figure 3.** Increased transcriptional activity of markers and mediators of the adaptive immunity in trabectedin-treated tumors. Real-time PCR of genes coding for: CD3, CD4, CD8, FOXP3, Granzyme B, Perforin, MX1, CXCL10, IFN- $\gamma$ , PD-1, and PDL-1. Graphs represent the mean  $\pm$  SEM of four individual tumor samples per each group of mice (two different experiments, two mice per group per experiment). Statistical analysis: \* $p$  < 0.05, \*\* $p$  < 0.01 (unpaired t-test with Welch's correction).

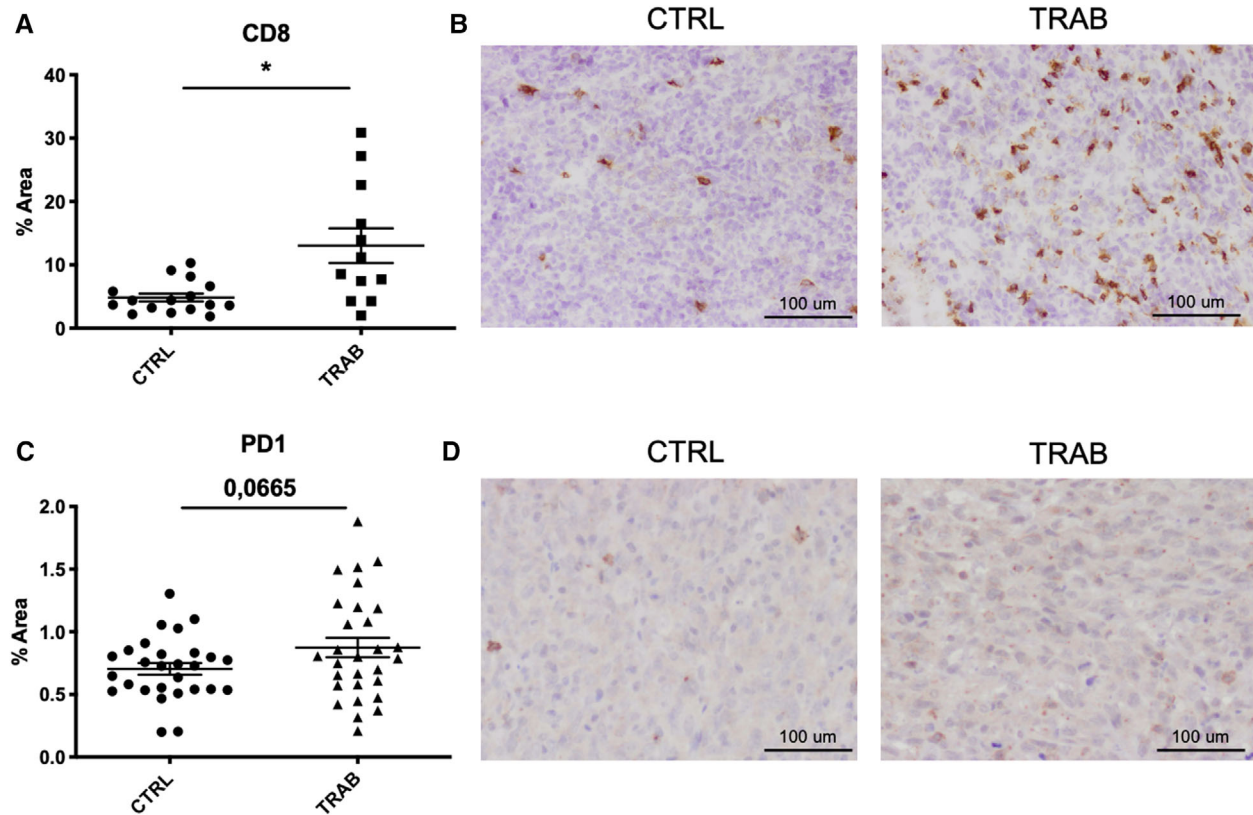
of mice treated with trabectedin. As observed in the flow cytometry analysis, the genes coding for CD3 and CD8 were significantly upregulated in treated tumors (Fig. 3A and C), while CD4 and FOXP3 did not show a significant variation (Fig. 3B and D). The genes coding for the serine protease granzyme B and the pore-forming protein perforin, two cytotoxic molecules produced by CD8<sup>+</sup> T lymphocytes, were also more expressed (Fig. 3E and F). mRNA levels of IFN- $\gamma$  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<sup>+</sup> 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 environ-

ment 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 $\times$ .

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.

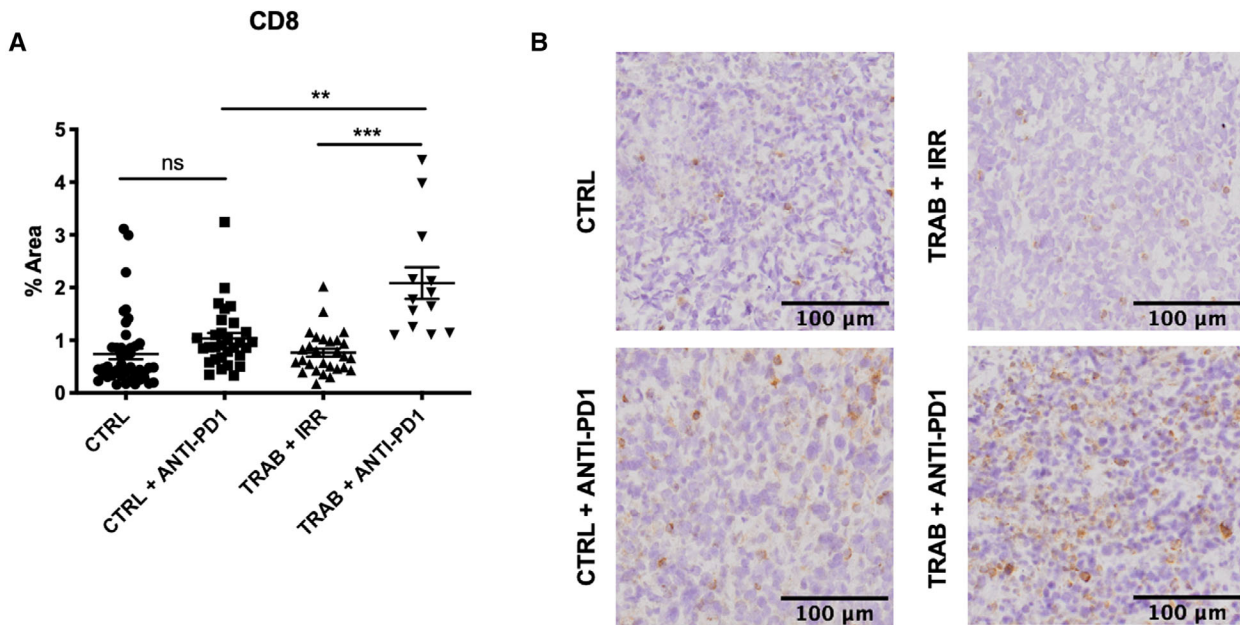
## Discussion

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- $\gamma$ , 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 6.** Infiltration of CD8<sup>+</sup> 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).

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 rationale 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); CD115 PE (eBioscience, San Diego, CA, USA; clone AFS98; catalogue number 12-1152-82) or F4/80 PE (Bio-Rad, Hercules, CA, USA; catalogue number MCA497PE); CD3 PeCy7 (BD Biosciences; clone 145-2C11; catalogue number 552774), CD19 FITC (BD Biosciences; clone 1D3; catalogue number 557398), Ly6G PE-Cy7 (BD Biosciences; clone 1A8; catalogue number 560601), and Ly6C FITC (BD Biosciences; clone AL-21; catalogue number 553104).



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 APC 780 (eBioscience; clone PC61.5; catalogue number: 47-0251-82).

Labeled cells were fixed in PBS +1% formalin. Acquisition was performed at FACS CANTO II instrument (BD Biosciences) and analyzed by FACS Diva and FlowJo software version 6.1.1 (BD Biosciences).

### 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:

$$\Delta CT = CT (\text{a target gene}) - CT (\text{GAPDH}).$$

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

$$2^{-\Delta\Delta CT}, \text{ where } -\Delta\Delta CT \text{ is } \Delta CT (\text{treated sample}) - \Delta CT (\text{average of control sample}).$$

### 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% H<sub>2</sub>O<sub>2</sub> for 5 min and then rodent block M was used to block unspecific binding sites. Sections were incubated with Biotin Rat Anti-Mouse CD8 (Invitrogen, 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

**Table 1.** Primers

mCD3 For	5' ATGCGGTGGAACACTTTCTGG 3'
mCD3 Rev	5' GCACGTCAACTCTACACTGGT 3'
mCD4 For	5' AGGTGATGGGACCTACCTCTC 3'
mCD4 Rev	5' GGGGCCACCACTTGAACCTAC 3'
mCD8 For	5' CTCTGGCTGGTCTTCAGTATGA 3'
mCD8 Rev	5' TCTTTGCCGTATGGTTGGTTT 3'
mGAPDH For	5' CCTTGACTGTGCCGTTGAATTT 3'
mGAPDH Rev	5' GCAAAGTGGGAGATTGTTGCCAT 3'
mFOXP3 For	5' CCCATCCCAGGAGTCTTG 3'
mFOXP3 Rev	5' ACCATGACTAGGGGCACTGTA 3'
mGranzymeB For	5' CCACTCTCGACCCTACATGG 3'
mGranzymeB Rev	5' GGCCCCAAAAGTGACATTTATT 3'
mPerforin For	5' AGCACAAGTTCGTGCCAGG 3'
mPerforin Rev	5' GCGTCTCTCATTAGGGAGTTT 3'
mIFNγ For	5' GGAAGTGGCAAAGGATGGTG 3'
mIFNγ Rev	5' ATGTTGTTGCTGATGGCCTG 3'
mMX1 For	5' GACCATAGGGGTCTTGACCAA 3'
mMX1 Rev	5' AGACTTGCTCTTTCTGAAAAGCC 3'
mCXCL10 For	5' CCAAGTGCTGCCGTCAATTTTC 3'
mCXCL10 Rev	5' GGCTCGCAGGGATGATTTCAA 3'
mPD-1 For	5' ACCCTGGTCATTCACTTGGG 3'
mPD-1 Rev	5' CATTGCTCCCTCTGACACTG 3'
mPDL1 For	5' GCTCCAAAGGACTTGTACGTG 3'
mPDL1 Rev	5' TGATCTGAAGGGCAGCATTTTC 3'

Buffer 1X (DV2005L2J Biocare Medical) (3 min at 125°C, 5 min at 90°C) (PD1, F4/80); antigen unmasking for CD8 (paraffin) was performed with temperature bath (20 min at 98°C) in EDTA pH 8.00 buffer 0.25 mM. Endogenous peroxidases were blocked with 2% H<sub>2</sub>O<sub>2</sub> for 20 min and then rodent block M (for CD8 staining—paraffin, and for F4/80) or PBS/BSA 2% (for PD1 staining) were used to block unspecific binding sites. Sections were incubated with the following antibodies: rabbit antimouse PD1 (Sino Biological, cat. n. 50124-RP02), rat antimouse CD8 (Invitrogen), and rat antimouse F4/80 (AbD Serotec, cat.no. MCA497G). All the primary antibodies were incubated for 1 h in humid chamber at room temperature. As secondary antibody we used: Rat on Mouse HRP polymer kit (Biocare Medical) (CD8, F4/80), Mach1 (Biocare Medical) (PD1). 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

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.



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**Authors contribution:** Study supervision, research design, and writing the manuscript: CB, RF, PA, M. D'I.; performing in vitro experiments and acquisition of data: CB, ML, ED, FSC; in vivo experiment and acquisition of data: CB, RF, ML, MM.

**Conflict of interest:** The authors declare no potential conflict of interest.

**Data availability statement:** The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Peer review:** The peer review history for this article is available at <https://publons.com/publon/10.1002/eji.202149379>

## References

- Sharma, P. and Allison, J. P. Dissecting the mechanisms of immune checkpoint therapy. *Nat. Rev. Immunol.* 2020. 20: 75–76.
- Bronte, V., Brandau, S., Chen, S. H., Colombo, M. P., Frey, A. B., Greten, T. F., Mandruzzato, S. et al., Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat. Commun.* 2016. 7: 12150.
- Sharma, P., Hu-Lieskovan, S., Wargo, J. A. and Primary, R. A., Adaptive and acquired resistance to cancer immunotherapy. *Cell* 2017. 168: 707–723.
- Anfray, C., Ummano, A., Andon, F. T. and Allavena, P. Current strategies to target tumor-associated-macrophages to improve anti-tumor immune responses. *Cells* 2019. 9: 46.
- Belgiovine, C., Digifico, E., Anfray, C. and Ummano, A., Torres Andon, F. Targeting tumor-associated macrophages in anti-cancer therapies: Convincing the traitors to do the right thing. *J. Clin. Med.* 2020. 9: 3226.
- Beltraminelli, T. and De Palma, M. Biology and therapeutic targeting of tumour-associated macrophages. *J. Pathol.* 2020. 250: 573–592.
- DeNardo, D. G. and Ruffell, B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 2019. 19: 369–382.
- Lebgege, E., Arnouk, S. M., Bardet, P. M. R., Kiss, M., Raes, G. and Van Ginderachter, J. A. Innate immune defense mechanisms by myeloid cells that hamper cancer immunotherapy. *Front. Immunol.* 2020. 11: 1395.
- Lopez-Yrigoyen, M., Cassetta, L. and Pollard, J. W. Macrophage targeting in cancer. *Ann. N. Y. Acad. Sci.* 2020.1499: 18–41.
- Mantovani, A., Marchesi, F., Malesci, A., Laghi, L. and Allavena, P. Tumour-associated macrophages as treatment targets in oncology. *Nat. Rev. Clin. Oncol.* 2017. 14: 399–416.
- Yang, M., McKay, D., Pollard, J. W. and Lewis, C. E. Diverse functions of macrophages in different tumor microenvironments. *Cancer Res.* 2018. 78: 5492–5503.
- Cannarile, M. A., Weisser, M., Jacob, W., Jegg, A. M., Ries, C. H. and Ruttiger, D. Colony-stimulating factor 1 receptor (CSF1R) inhibitors in cancer therapy. *J. Immunother. Cancer.* 2017. 5: 53.
- Pyonteck, S. M., Akkari, L., Schuhmacher, A. J., Bowman, R. L., Sevenich, L., Quail, D. F., Olson, O. C. et al., CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat. Med.* 2013. 19: 1264–1272.
- Belgiovine, C., D'Incalci, M., Allavena, P. and Frapolli, R. Tumor-associated macrophages and anti-tumor therapies: complex links. *Cell. Mol. Life Sci.* 2016. 73: 2411–2424.
- D'Incalci, M., Badri, N., Galmarini, C. M. and Allavena, P. Trabectedin, a drug acting on both cancer cells and the tumour microenvironment. *Br J Cancer (Review).* 2014. 111: 646–650.
- D'Incalci, M. and Galmarini, C. M. A review of trabectedin (ET-743): a unique mechanism of action. *Mol Cancer Ther.* 2010. 9: 2157–2163.
- Larsen, A. K., Galmarini, C. M. and D'Incalci, M. Unique features of trabectedin mechanism of action. *Cancer Chemother. Pharmacol.* 2016. 77: 663–671.
- Andreeva-Gateva, P. and Chakar, S. The place of trabectedin in the treatment of soft tissue sarcoma: an umbrella review of the level one evidence. *Expert Opin. Orphan Drugs.* 2019. 7: 105–115.
- Colombo, N., Hardy-Bessard, A. C., Ferrandina, G., Marth, C. and Romero, I. Experience with trabectedin + pegylated liposomal doxorubicin for recurrent platinum-sensitive ovarian cancer unsuited to platinum rechallenge. *Expert Rev. Anticancer Ther.* 2016. 16: 11–19.
- Grignani, G., D'Ambrosio, L., Pignochino, Y., Palmerini, E., Zucchetti, M., Boccone, P., Aliberti, S. et al., Trabectedin and olaparib in patients with advanced and non-resectable bone and soft-tissue sarcomas (TOMAS): an open-label, phase 1b study from the Italian Sarcoma Group. *Lancet Oncol.* 2018. 19: 1360–1371.
- Grosso, F., Jones, R. L., Demetri, G. D., Judson, I. R., Blay, J. - Y., Le Cesne, A., Sanfilippo, R. et al., Efficacy of trabectedin (ecteinascidin-743) in advanced pretreated myxoid liposarcomas: a retrospective study. *Lancet Oncol.* 2007. 8: 595–602.
- Palmerini, E., Sanfilippo, R., Grignani, G., Buonadonna, A., Romanini, A., Badalamenti, G., Ferraresi, V. et al., Trabectedin for patients with advanced soft tissue sarcoma: a non-interventional, retrospective, multicenter study of the Italian sarcoma group. *Cancers (Basel)* 2021. 13: 1053.
- Assi, T., Kattan, J., El Rassy, E., Honore, C., Dumont, S., Mir, O. and Le Cesne, A. A comprehensive review of the current evidence for trabectedin in advanced myxoid liposarcoma. *Cancer Treat. Rev.* 2019. 72: 37–44.
- Di Giandomenico, S., Frapolli, R., Bello, E., Uboldi, S., Licandro, S. A., Marchini, S., Beltrame, L. et al., Mode of action of trabectedin in myxoid liposarcomas. *Oncogene* 2014. 33: 5201–5210.
- Allavena, P., Signorelli, M., Chieppa, M., Erba, E., Bianchi, G., Marchesi, F., Olimpio, C. O. et al., Anti-inflammatory properties of the novel antitumor agent yondelis (trabectedin): inhibition of macrophage differentiation and cytokine production. *Cancer Res.* 2005. 65: 2964–2971.
- Germano, G., Frapolli, R., Belgiovine, C., Anselmo, A., Pesce, S., Liguori, M., Erba, E. et al., Role of macrophage targeting in the antitumor activity of trabectedin. *Cancer Cell* 2013. 23: 249–262.
- Liguori, M., Buracchi, C., Pasqualini, F., Bergomas, F., Pesce, S., Sironi, M., Grizzi, F. et al., Functional TRAIL receptors in monocytes and tumor-associated macrophages: a possible targeting pathway in the tumor microenvironment. *Oncotarget* 2016. 7: 41662–41676.
- Germano, G., Frapolli, R., Simone, M., Tavecchio, M., Erba, E., Pesce, S., Pasqualini, F. et al., Antitumor and anti-inflammatory effects of trabectedin on human myxoid liposarcoma cells. *Cancer Res.* 2010. 70: 2235–2244.
- Borgoni, S., Iannello, A., Cutrupi, S., Allavena, P., D'Incalci, M., Novelli, F. and Cappello, P. Depletion of tumor-associated macrophages switches the epigenetic profile of pancreatic cancer infiltrating T cells and restores their anti-tumor phenotype. *Oncoimmunology* 2018. 7: e1393596.

- 30 Carminati, L., Pinessi, D., Borsotti, P., Minoli, L., Giavazzi, R., D'Incalci, M., Belotti, D. et al., Antimetastatic and antiangiogenic activity of trabectedin in cutaneous melanoma. *Carcinogenesis*. 2019. **40**: 303–312.
- 31 Denton, N. L., Chen, C. Y., Hutzen, B., Currier, M. A., Scott, T., Nartker, B., Leddon, J. L. et al., Myelolytic treatments enhance oncolytic herpes virotherapy in models of ewing sarcoma by modulating the immune microenvironment. *Mol. Ther. Oncolytics*. 2018. **11**: 62–74.
- 32 Jones, J. D., Sinder, B. P., Paige, D., Soki, F. N., Koh, A. J., Thiele, S., Shiozawa, Y. et al., Trabectedin reduces skeletal prostate cancer tumor size in association with effects on M2 macrophages and efferocytosis. *Neoplasia*. 2019. **21**: 172–184.
- 33 Ratti, C., Botti, L., Cancila, V., Galvan, S., Torselli, I., Garofalo, C., Manara, M. C. et al., Trabectedin overrides osteosarcoma differentiative block and reprograms the tumor immune environment enabling effective combination with immune checkpoint inhibitors. *Clin. Cancer Res*. 2017. **23**: 5149–5161.
- 34 Povo-Retana, A., Mojena, M., Stremtan, A. B., Fernández-García, V. B., Gómez-Sáez, A., Nuevo-Tapióles, C., Molina-Guijarro, J. M. et al., Specific effects of trabectedin and lurbinectedin on human macrophage function and fate-novel insights. *Cancers (Basel)* 2020. **12**:3060.
- 35 Banerjee, P., Zhang, R., Ivan, C., Galletti, G., Clise-Dwyer, K., Barbaglio, F., Scarfó, L. et al., Trabectedin reveals a strategy of immunomodulation in chronic lymphocytic leukemia. *Cancer Immunol. Res*. 2019. **7**: 2036–2051.
- 36 Casagrande, N., Borghese, C., Favero, A., Vicenzetto, C. and Aldinucci, D. Trabectedin overcomes doxorubicin-resistance, counteracts tumor-immunosuppressive reprogramming of monocytes and decreases xenograft growth in Hodgkin lymphoma. *Cancer Lett* 2021. **500**: 182–193.
- 37 Romano, M., Della Porta, M. G., Galli, A., Panini, N., Licandro, S. A., Bello, E., Craparotta, I. et al., Antitumour activity of trabectedin in myelodysplastic/myeloproliferative neoplasms. *Br. J. Cancer* 2017. **116**: 335–343.
- 38 Guo, Z., Wang, H., Meng, F., Li, J. and Zhang, S. Combined trabectedin and anti-PD1 antibody produces a synergistic antitumor effect in a murine model of ovarian cancer. *J. Transl. Med*. 2015. **13**: 247.
- 39 Cossarizza, A., Chang, H. D., Radbruch, A., Acs, A., Adam, A., Adam-Klages, S., Agace, W. et al., Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur. J. Immunol*. 2019. **49**: 1457–1973.

**Abbreviations:** IC: immunocompetent · TAMs: tumor-associated macrophages; MX1, myxoma resistance protein 1 · MX1: myxoma resistance protein 1

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