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Lurbinectedin reduces tumour-associated macrophages and the inflammatory tumour microenvironment in preclinical models

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Background: Lurbinectedin is a novel anticancer agent currently undergoing late-stage (Phase II /III) clinical evaluation in platinum-resistant ovarian, BRCA1/2-mutated breast and small-cell lung cancer. Lurbinectedin is structurally related to trabectedin and it inhibits active transcription and the DNA repair machinery in tumour cells.

Methods: In this study we investigated whether lurbinectedin has the ability to modulate the inflammatory microenvironment and the viability of myeloid cells in tumour-bearing mice.

Results: Administration of lurbinectedin significantly and selectively decreased the number of circulating monocytes and, in tumour tissues, that of macrophages and vessels. Similar findings were observed when a lurbinectedin-resistant tumour variant was used, indicating a direct effect of lurbinectedin on the tumour microenviroment. *In vitro*, lurbinectedin induced caspase-8-dependent apoptosis of human purified monocytes, whereas at low doses it significantly inhibited the production of inflammatory/growth factors (CCL2, CXCL8 and VEGF) and dramatically impaired monocyte adhesion and migration ability. These findings were supported by the strong inhibition of genes of the Rho-GTPase family in lurbinectedin-treated monocytes.

Conclusions: The results illustrate that lurbinectedin affects at multiple levels the inflammatory microenvironment by acting on the viability and functional activity of mononuclear phagocytes. These peculiar effects, combined with its intrinsic activity against cancer cells, make lurbinectedin a compound of particular interest in oncology.

In the last decade, our knowledge on the tumour microenviroment (TME) has considerably increased and the current evidence is that reciprocal interactions between cancer and stromal cells are of pivotal importance for the survival of tumour cells and disease progression (Knowles and Harris, 2007; Mantovani *et al*, 2008; Allavena and Mantovani, 2012; Coussens *et al*, 2013; Noy and Pollard, 2014). The TME is the site of the cancer-promoting inflammation, now a recognised hallmark of cancer (Hanahan and Weinberg, 2011). Activated fibroblasts, endothelial cells and innate immunity cells concur to build up a reactive and deregulated environment, where several biological mediators and matrix-degrading enzymes are continuously produced, eventually favouring tumour growth. In this scenario, tumour-associated macrophages (TAM) have a prominent role, as they produce several growth factors for tumour cells and the vessel network, and inflammatory cytokines and chemokines that amplify the

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tumour-associated inflammation (Knowles and Harris, 2007; Noy and Pollard, 2014; Mantovani and Allavena, 2015; Belgiovine *et al*, 2016). Strong evidence demonstrates that in most tumours, TAM stimulate tumour progression and limit therapeutic responses (Kryczek *et al*, 2006; De Palma and Lewis, 2013; Galluzzi *et al*, 2014; Mantovani and Allavena, 2015; Belgiovine *et al*, 2016). Furthermore, TAM have strong immunosuppressive functions: they have been described to directly invalidate antitumour T-cell activity by suppressing CD8+ T-cell proliferation and IFNγ expression through programmed death ligand 1 (Mantovani *et al*, 2005; Kryczek *et al*, 2006; Kuang *et al*, 2009; Bloch *et al*, 2013). Notably, the response rates in the PD-1/PD-L1 trials relate, at least partially, to PD-L1 expression in the stroma (Herbst *et al*, 2014; Tumeh *et al*, 2014; Zhu *et al*, 2014; Qu *et al*, 2016), consistent with a role for macrophages and/or other stromal cells in blocking antitumour T-cell responses. Therefore, drugs affecting the TME are regarded with particular interest in the oncology field. Already available examples include anti-angiogenic drugs, immune checkpoints inhibitors and, more recently, drugs targeting macrophages, such as kinase inhibitors or antibodies directed to the CSF-1 receptor (Zeisberger *et al*, 2006; Priceman *et al*, 2010; DeNardo *et al*, 2011; Hume and MacDonald, 2011; Pyonteck *et al*, 2013; Ries *et al*, 2014). These drugs have recently entered the clinic with much expectation. However, strategies solely targeting tumour macrophages are unlikely to be successful and combination treatments with conventional or biological therapies are under investigation to improve the anti-tumour efficacy.

Lurbinectedin (PM01183) is an analogue of trabectedin that has shown very promising clinical activity in a broad range of clinical trials and is currently under investigation in pivotal phase III studies in patients with platinum-resistant ovarian cancer (NCT02421588) and small-cell lung cancer (NCT02566993). In tumour cells, lurbinectedin inhibits active transcription through the following: (1) its binding to CG-rich sequences, mainly located around promoters of protein coding genes; (2) the irreversible stalling of elongating RNA polymerase II on the DNA template and its specific degradation by the ubiquitin/proteasome machinery; and (3) the generation of XPF-dependent single-strand and double-strand DNA breaks, and subsequent apoptosis (Elez *et al*, 2014; Moneo *et al*, 2014; Pernice *et al*, 2016; Santamaria Nunez *et al*, 2016). In addition, lurbinectedin is extremely effective against cancer cells with impairment of homologous recombination repair (Romano *et al*, 2013). However, the role of lurbinectedin on the TME is unknown.

Our previous studies reported that trabectedin is a unique example of an antitumour agent impacting both on cancer cells and on the TME (Germano *et al*, 2010; Germano *et al*, 2013). We demonstrated that trabectedin induces caspase-dependent apoptosis specifically in the monocyte-macrophage lineage and this effect is remarkably selective as other leukocyte subsets are not affected (Germano *et al*, 2013). We further found that trabectedin significantly inhibits the transcription of several biological mediators, including CCL2, CXCL8, IL-6 and VEGF, which have an important role in cancer-promoting inflammation (Germano *et al*, 2010). These effects of trabectedin on mononuclear phagocytic cells were confirmed *in vivo* in mouse tumour models and in treated patients, and likely constitute an important determinant of its antitumour efficacy (Germano *et al*, 2013). In this study, we addressed the question whether lurbinectedin shares similar modulating effects on the tumour microenvironment. Our results demonstrate that lurbinectedin significantly reduces monocyte viability by inducing apoptotic cell death. In mouse tumour models, lurbinectedin decreases the number of circulating monocytes and tumour macrophages, inhibits angiogenesis and restrains tumour growth, even when cancer cells were resistant to its direct effect. A global gene expression analysis of human monocytes treated with lurbinectedin revealed a previously unidentified strong inhibition of the Rho GTPase pathway, which significantly impacts on monocyte-macrophage functional activities.

MATERIALS AND METHODS

Drugs. Lurbinectedin and trabectedin (PharmaMar, Colmenar Viejo, Madrid, Spain) were dissolved in DMSO to 1 mM and kept at −20 °C. Doxorubicin (Sigma) was dissolved in water to 2 mM and kept −20 °C.

Human monocytes *in vitro* studies. Human monocytes were obtained from the leukocyte-rich component (buffy coat) of blood healthy donors along a protocol approved by the Ethical committee of Humanitas Institute. Before blood donation, volunteers sign an informed consent at the Transfusion Centre (San Matteo Hospital, Pavia, Italy) where blood is collected. The informed consent clearly specifies that their blood donation can be used for research activity.

Monocytes were purified through density gradients, as described (Liguori *et al*, 2016). Cells were treated with lurbinectedin or trabectedin (2.5–10 nM) for different time points as indicated. Cell death was quantified with propidium iodide staining; caspase-dependent apoptosis was evaluated as caspase 8 activation on permeabilised cells in flow cytometry (Germano *et al*, 2013). Cytokine production was quantified by ELISA in the supernatants of LPS-stimulated monocytes pretreated with lurbinectedin or trabectedin at non-cytotoxic concentration (5 nM for 1 h). Cells were then washed, stimulated with LPS (Alexis) at 1 μg ml^{-1} and supernatants collected after 24 h incubation at 37 °C. Monocyte migration was performed in Transwell as described (Germano *et al*, 2013). Cells were pre-treated under non-cytotoxic conditions, washed, incubated for 5 h in medium with 1% FBS and then seeded in the upper compartment of Transwell. CCL2 (100 ng ml^{-1}) was placed in the lower compartment. After incubation at 37 °C for 90 min, migrated cells were counted in the lower filter face and in the well. Cell adhesion in pretreated cells was investigated as the number of plastic-adherent cells after 1 h incubation at 37 °C.

Microarray analysis and RT-PCR validation analysis. Human monocytes were pre-treated for 1 h with 10 nM lurbinectedin or trabectedin or doxorubicin (1 μM) and then stimulated with LPS (100 ng ml^{-1}) for 6 h. Total RNA was extracted and purified using a commercial available kit (miRNAesy Qiagen, Milano, Italy); this step was in part mechanised using an automatic extraction system (Qiacube, Qiagen). The amount of total RNA was determined by an innovative high-speed microfluidic UV/VIS spectrophotometer QIAxpert (Qiagen), and the integrity and quality of RNA was evaluated by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

For microarray experiments, 150 ng of RNA were labelled with Cy3 using LowInput QuickAmp labelling kit (Agilent Technologies, Santa Clara, CA, USA) and hybridised according to the manufacturer's instructions. After 20 h incubation at 65 °C in rotation at 20 r.p.m., arrays were washed and scanned with a laser confocal scanner (G2565B, Agilent Technologies, Santa Clara, USA).

Data obtained from the scanner were pre-processed, discarding probes that were marked as unreliable by Feature Extraction in at least 60% of the samples. The resulting filtered data set was then normalised using the 'quantile' method, without subtracting background intensities. Differential expression was calculated with the Rank Product method (Breitling *et al*, 2004), calling genes differentially expressed if their percentage of false positives was <5%.

Functional enrichment was calculated with the 'impact factor' method as implemented in the graphite R package (Draghici *et al*,

Table 1. Primers used for gene analysis

Gene	Amplicon length	FW (5′–3′)	RV (5′–3′)
Human			
GAPDH	230	aggtcggagtcaacggattt	ATCTCGCTCCTGGAAGATGG
G6PD	76	tgcccccgaccgtctac	atgcggttccagcctatctg
B2M	69	aagcagcatcatggaggttt	agcaagcaagcagaatttgg
PPIA	301	gcgtctccttttgagctgttt	gtcttggcagtgcagatgaa
ARHGAP 26	113	ccgggaacctgtctacaact	ctctggtttccacagcatgg
ARHGAP 32	101	agcatgtgaaaaagttacccttca	aagctgtatgctgccgaact
MYO9A	87	ttcgtagatcaatgggaaagg	acacagaaggcaaccgaact
VAV3	87	ggcagggcagaaatttagca	ctactggtttgggcacacat
SRGAP 2	103	acaagccaagcatgaccttc	gctggagtcctgtttcctca
Murine			
GAPDH	91	cccagcttaggttcatcagg	tacggccaaatccgttcac
G6PD	116	acgccctcttgcgttaaat	tctgccatgatgttttctgagtt
B2M	70	ctcggtgaccctggtcttt	ttgaggggttttctggatagc
PPIA	80	tcaaccccaccgtgttctt	tttgtctgcaaacagctcgaa
ARHGAP 26	110	gagacgctcaagtcacacga	gccgaagacaaattcttgag
ARHGAP 32	122	ggaaacactcagcgcaatg	tgaagggtaacttcttcacatgct
MYO9A	72	ttctgagccggattgcttc	atggccaactgtaagcgttc
VAV3	141	cccagcaacctaaatcacaa	ccgccgtcagaaatctttt
SRGAP 2	130	gagttggcggagtctcctta	gttcactagcctggctctgc

2007; Sales *et al*, 2012) over two pathway repositories (KEGG and Reactome). The *P*-values from the test were corrected for multiple testing with the false discovery rate (Benjamini and Hochberg, 1995). In accordance to the MIAME guidelines, array data files have been submitted to Array Express (ID E-MTAB-5366).

RT-PCR analysis. Five hundred nanograms of total RNA was reverse transcribed using the High-Capacity cDNA Archive Kit following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA), to assess the differential expression of genes in control-stimulated cells and cells treated with different drugs by quantitative real-time PCR. mRNA expression was analysed using QuantiFast SYBR Green PCR Master Mix (Qiagen) and dedicated primers that were designed (Table 1). Data were normalised using geometric mean of four independent house-keeping genes (*cyclophilin A*, *GAPDH*, *G6PD* and *B2M*). Experiments were run in triplicate for each case to assess technical variability, using an automatic liquid handling station (QIAgility, Qiagen), on Rotor-Gene Q (Qiagen). Analysis was performed by using the $2-\Delta\Delta Ct$ protocol and expressed as fold changes (arbitrary unit) compared with control-stimulated cells, set as 1.

Mouse tumour models. Procedures involving animals and their care were conducted in conformity with the following laws, regulations and policies governing the care and use of laboratory animals: Italian Governing Law (D.lgs 26/2014, authorisation number19/2008-A issued 6 March 2008 by Ministry of Health); Mario Negri Institutional Regulations and Policies providing internal authorisation for persons conducting animal experiments (Quality Management System Certificate—UNI EN ISO 9001 : 2008—registration number 6121); the NIH Guide for the Care and Use of Laboratory Animals (2011 edition); EU directives and guidelines (EEC Council Directive 2010/63/UE), and in line with Guidelines for the welfare and use of animals in cancer research (Workman *et al*, 2010). Animal experiments has been reviewed and approved by the IRFMN Animal Care and Use Committee that includes members '*ad hoc*' for ethical issues. Animals were housed in the Institute's Animal Care Facilities, which meet international standards; they are regularly checked by a certified veterinarian who is responsible for health monitoring, animal welfare supervision, experimental protocols and procedures revision.

C57/BL/6J mice between 6 and 10 weeks of age were purchased from Charles River (Calco, Como, Italy). The transplantable MN/MCA1 mouse fibrosarcoma and the trabectedin-resistant variant MN/MCA1-RES were used. The MN/MCA1-RES variant was generated by *in vitro* exposure to stepwise increasing concentrations of trabectedin, until stable resistance was achieved. Resistance was verified as drug susceptibility (apoptosis) *in vitro* and in tumour cells *ex vivo* after growth in mice (Germano *et al*, 2013). Tumour cells were inoculated i.m. (10^5 cells); treatments (10 mice per group) started when tumours were palpable.

Lurbinectedin and trabectedin were used at the doses of 0.13 and 0.10 mg kg^{-1}, respectively (sub-optimal doses) in the wild-type MN/MCA1 model and at the doses of 0.2 and 0.15 mg kg^{-1}, respectively in the MN/MCA1-RES model. These treatments did not completely abrogate tumour growth. This choice was made to optimally study the tumour microenvironment. Drugs were administered i.v. once a week for 3 weeks (q7d × 3). The tumour growth inhibition %T/C (treated/control × 100) ratio was used to quantify treatment effects of the drugs under study.

Analysis of the tumour microenvironment. Blood cells were collected from the eye vein of anaesthetised mice; splenocytes from disaggregated spleens were filtered through Falcon strainers. Mouse tumours were cut into small pieces, disaggregated with collagenase (0.5 mg ml^{-1}) and filtered through strainers. Cells (10^6) were stained with live and death dye (Invitrogen, Carlsbad, CA, USA), and with the following specific antibodies: PerCp- Rat CD45 (30F11), APCRat CD11b (M1/70), PE Hamster CD3 (145-2C11), PE Rat Ly6C and Ly6C (RB6-8C5), FITC Rat Ly6C (AL-21), PE Rat Ly-6G (1A8), as well as relative control antibody Alexa Fluor 647 CD19 (1D3) (eBioscience, Waltham, MA, USA). PacificBlue Annexin V was purchased from Invitrogen, and streptavidin dye-conjugated (APC) and BD TruCOUNT tubes were obtained from BD Biosciences (San Jose, CA, USA). Flow cytometry was performed by FACS CantoTM instrument and FACS Diva software version 6.1.1 (BD Biosciences).

Immunohistochemistry of mouse tumours was performed with anti-mouse PE F4/80 (A3-1, Serotec (Oxford, UK), anti-mouse CD31 (clone MEC13.3; BD Biosciences). In each experiment, five to eight tumours per group were analysed; results are the mean of positive cells in four slices per tumour. To perform analysis we used ImageJ programme.

Statistical analysis. Statistical analysis was performed using a paired Student's *t*-test. *P*-values of <0.05 were considered significant.

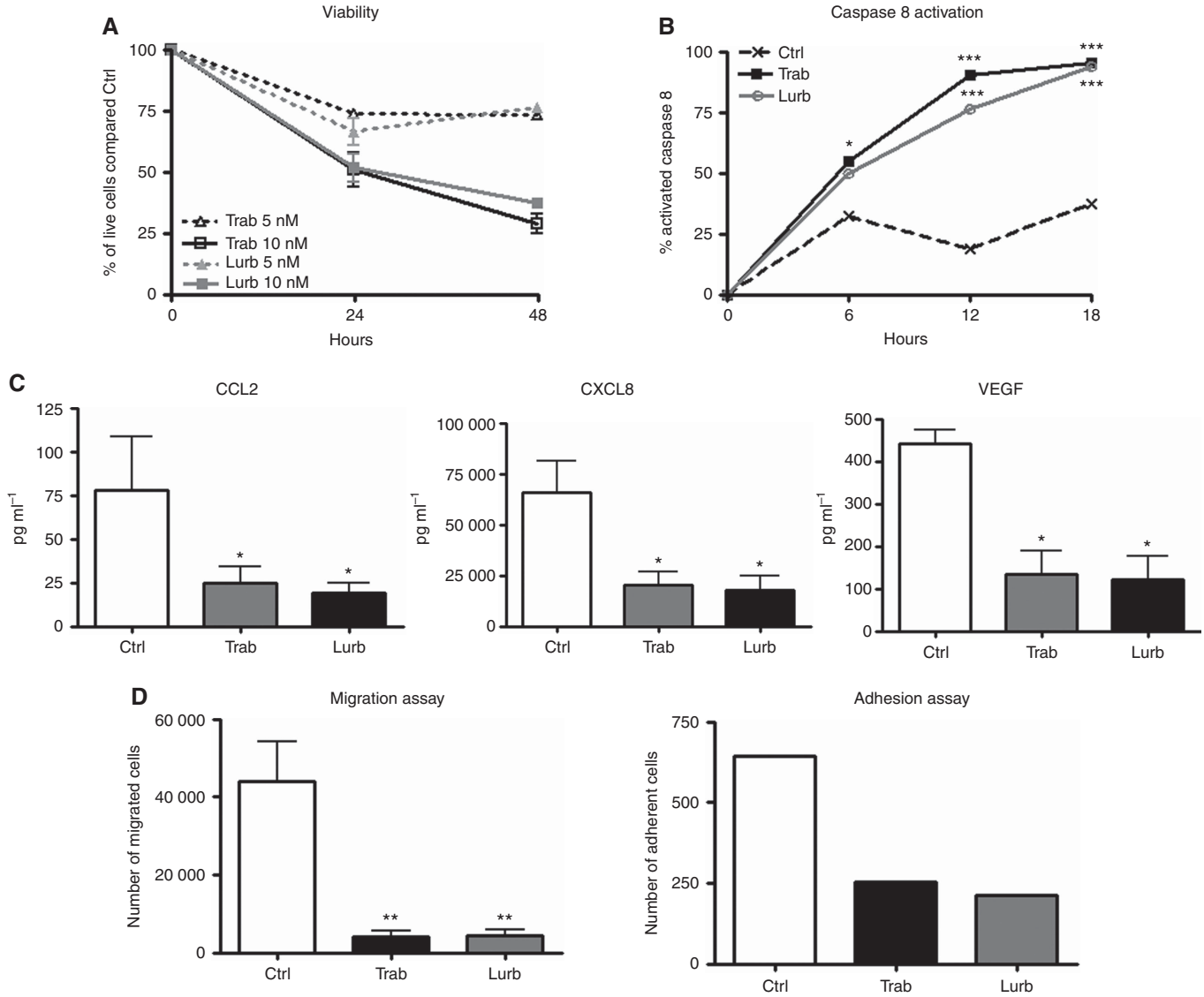

Figure 1. Effects of lurbinectedin on human purified monocytes *in vitro*. (**A**) Cell viability of monocytes treated with lurbinectedin or trabectedin at two different doses (5 and 10 nM) for up to 48 h, evaluated with Annexin/PI staining by flow cytometry; results are shown as % of live cells compared with untreated cells (mean ± s.e. of three experiments). (**B**) Caspase-8 activation in treated monocytes evaluated by flow cytometry with anti-cleaved CASP8 antibody. Caspase-8 activation peaks at 12 h and remains at plateau at 18 h. (**C**) Modulation of cytokine production (ELISA) by lurbinectedin and trabectedin in human LPS-stimulated monocytes. Monocytes were treated with drugs (5 nM) for 1 h, washed and cultured with fresh medium for 24 h. Both drugs significantly and similarly inhibited the production of CCL2, CXCL8 and VEGF. (**D**) Migration assay (left) and adhesion assay (right) of human monocytes pre-treated with lurbinectedin and trabectedin (5 nM), in response to CCL2 (100 ng ml^{-1}). Statistical analysis: *$P<0.05$, **$P<0.01$ and ***$P<0.001$ (Student's *t*-test).

RESULTS

Lurbinectedin induces apoptosis of human monocytes and affects their functional activity. We first verified the *in vitro* cytotoxic effect of lurbinectedin on human monocytes obtained from healthy donors. As shown in Figure 1A, treatment with different concentrations of lurbinectedin at different times significantly reduced monocyte viability in a concentration-dependent manner. At 24 h, 10 nM lurbinectedin caused 50% monocyte death, which raised to 75% after 48 h. The dose of 5 nM reduced viability of 25–30%. We checked the activation of caspase-8 in treated human monocytes by flow cytometry with an anti-caspase-8 antibody recognising its specific cleaved form. Lurbinectedin strongly activated caspase-8 with a peak at 12 h (Figure 1B). In both experiments, similar results were obtained with trabectedin (Figure 1A and B).

In order to evaluate the drug effect on the functional activity of monocytes, we tested a short treatment (1 h) with 5 nM lurbinectedin. This concentration was chosen to avoid the high levels of apoptosis observed with higher concentrations and longer incubation. As shown in Figure 1C, the production of specific inflammatory cytokines was inhibited in LPS-stimulated monocytes. The chemokines CCL2 and CXCL8, and the angiogenic factor VEGF were strongly reduced by both lurbinectedin and trabectedin (Figure 1C). To determine whether this effect was specific to monocytes or also occurred in tumour cells, we have tested the cytokines produced by the human myxoid liposarcoma cell line 402.91 using the same experimental non-cytotoxic conditions. The results showed that while CCL2 was strongly inhibited by drug treatment, CXCL8 was reduced to a lesser extent by lurbinectedin compared to LPS-stimulated monocytes (Supplementary Figure 1). Pentraxin-3 was also tested as

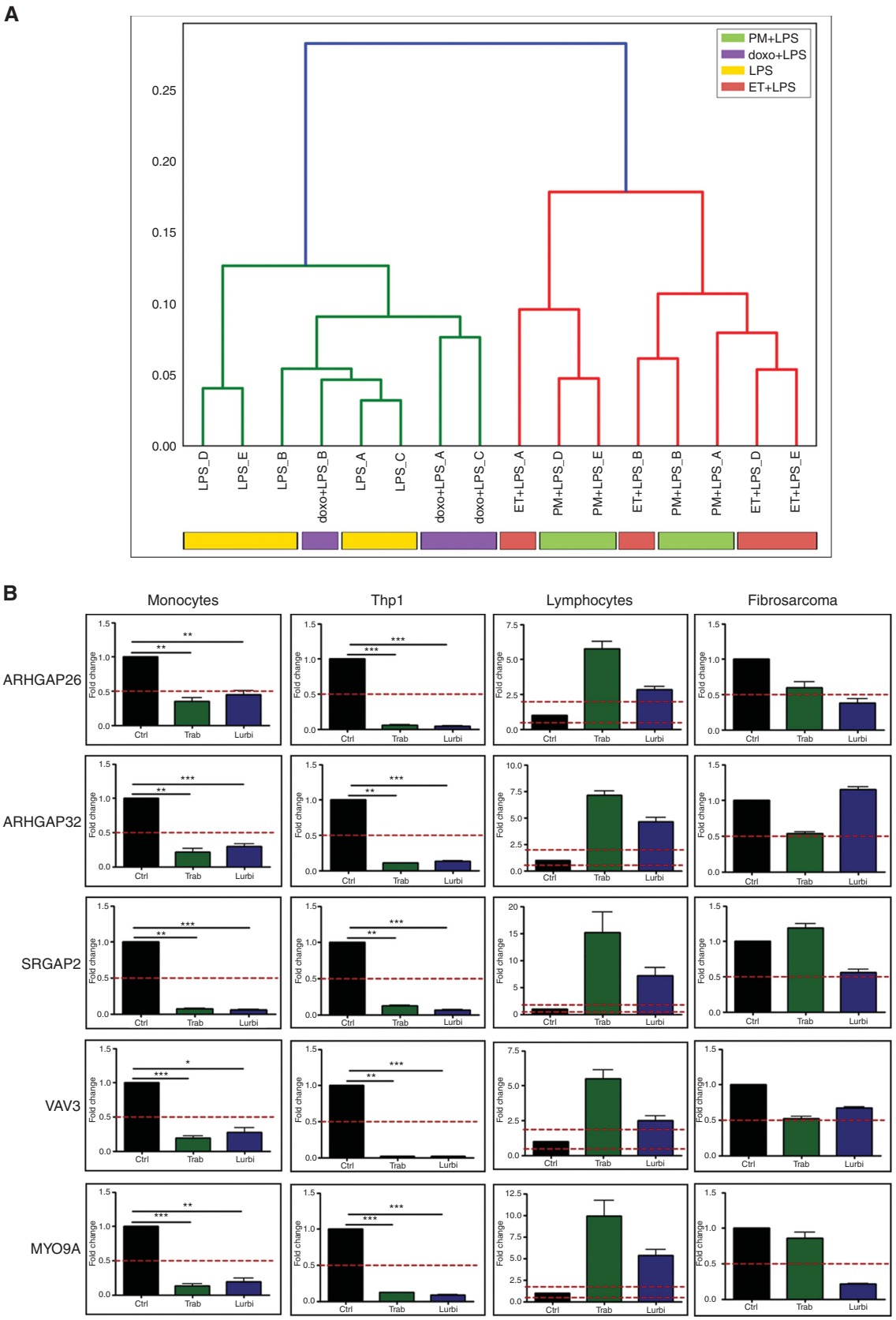

Figure 2. Modulation of gene expression by lurbinectedin in human LPS-stimulated monocytes. Monocytes were treated with lurbinectedin (10 nM), trabectedin (10 nM) or doxorubicin (1 μM) for 6 h (mean ± s.e. of five donors). (**A**) Dendogram plot showing the similarity of gene modulation for lurbinectedin (PM) and trabectedin (ET), in contrast with doxorubicin. (**B**) Modulation of Rho GTPase genes in human LPS-stimulated monocytes, in the myeloid cell line THP1, primary IL-2-activated human T lymphocytes and the murine fibrosarcoma MN/MCA1. Both lurbinectedin and trabectedin inhibit Rho GTPase genes selectively in myeloid cells. Statistical analysis: $*P<0.05$, $**P<0.01$ and $***P<0.001$ (Student's *t*-test). Data were analysed by the DDCT method and expressed as fold change (arbitrary unit) compared with untreated control (set as 1). Values below 0.5 or above 2 (red dashed lines) are genes differentially expressed.

liposarcoma cells are known high producers of this protein (Germano *et al*, 2010). Both drugs significantly attenuated its production (Supplementary Figure 1).

We further investigated the effect of drug treatment on the adhesion and migration ability of monocytes. A short treatment with lurbinectedin (5 nM for 1 h) had a dramatic effect on monocytes, significantly reducing their migration in response to the chemokine CCL2 and their adhesion to plastic surfaces (Figure 1D).

Global gene expression analysis of human monocytes. To have a broader view of the impact of lurbinectedin on monocytes, we performed a gene profiling analysis of LPS-stimulated monocytes pre-treated with lurbinectedin, with trabectedin for comparison or with doxorubicin as unrelated drug. A global analysis revealed that the transcriptomes modulated by lurbinectedin and trabectedin were similar to each other and distinct from that modulated by doxorubicin (Figure 2A). Among the genes down-modulated by trabectedin, 95% were also inhibited by lurbinectedin. One of the top affected pathways was that of the Rho GTPase family. Rho GTPases are crucial molecular switches that control many signalling events involved in diverse functions, such as actin-cytoskeleton organisation and cell motility (Etienne-Manneville and Hall, 2002). Several genes of the RhoGTPase family were significantly downregulated by both lurbinectedin and trabectedin. To support the robustness of our findings, RT-qPCR was used to validate expression changes in five genes members of this pathway (*arhgap26*, *arhgap32*, *srgap2*, *vav3* and *myo9a).* In monocytes as well as in myeloid leukaemia cells (THP-1, U937 and HL-60) these genes were significantly inhibited (Figure 2B and data not shown). In contrast, none of these genes were inhibited in primary activated T lymphocytes or in fibrosarcoma tumour cells (Figure 2B). These findings indicate specific selectivity of lurbinectedin and trabectedin for myeloid-derived cells and are in accordance with the results described above of impaired adhesion and migration ability of monocytes in functional assays.

***In vivo* anti-tumour efficacy of lurbinectedin and effects on the tumour microenvironment.** The results presented above with human monocytes raised the question whether the apoptotic-inducing effect of lurbinectedin has a role in its *in vivo* anti-tumour activity. This was addressed by testing a variant of the fibrosarcoma MN/MCA1 with stable resistance to trabectedin (MN/MCA1-RES) (Germano *et al*, 2013), which was confirmed to have cross-resistance with lurbinectedin (Supplementary Figure 2). In previous experiments we reported that the wild-type fibrosarcoma MN/MCA1 is highly susceptible to standard doses of trabectedin and lurbinectedin (0.15 and 0.2 mg kg^{-1}) with a treatment-to-control ratio T/C of 13% and 15%, respectively. Furthermore, spontaneous lung metastases were significantly reduced: untreated (median 45); trabectedin: (median 4); lurbinectedin: (median 2) (Romano *et al*, 2013). Thus, the two compounds showed similarly high anti-tumour activity.

In this study, to investigate the tumour microenvironment, we employed doses that did not completely abrogate tumour growth (i.e., 0.10 mg kg^{-1} of trabectedin and 0.13 mg kg^{-1} of lurbinectedin, i.v., q7d × 3, in the wild-type MN/MCA1 model; and 0.20 mg kg^{-1} of lurbinectedin and 0.15 mg kg^{-1} of trabectedin i.v., q7d × 3, in the resistant model). *In vivo* treatment of mice bearing the wild-type fibrosarcoma achieved a T/C of 43% and 40% (lurbinectedin and trabectedin) (Figure 3A). In mice bearing the resistant fibrosarcoma, T/C was 53% and 41.5% (Figure 3B).

A significant anti-tumour activity on cancer cells with demonstrated *in vitro* resistance to lurbinectedin is indicative of an effect on the tumour microenvironment. We therefore analysed circulating monocytes as well as tissue macrophages in tumour-bearing treated mice. After the first drug cycle, blood was collected

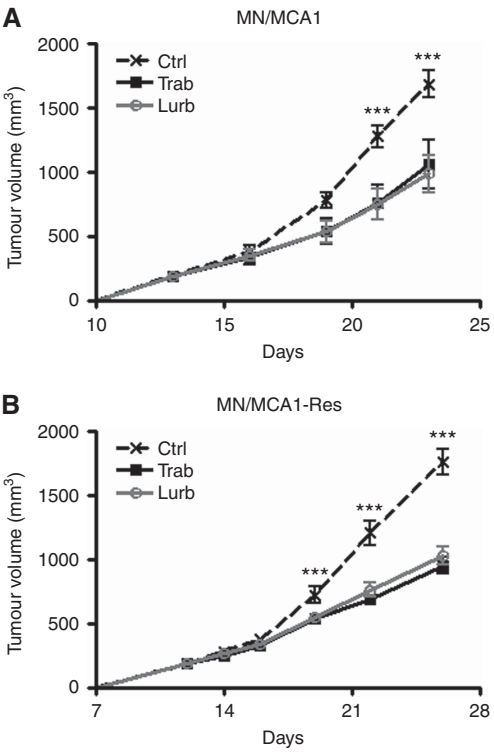

Figure 3. *In vivo* anti-tumour activity of lurbinectedin in mice. The syngenic fibrosarcoma MN/MCA1 (**A**) and its resistant variant RES-MN/MCA1 (**B**) were used. Mice were treated with lurbinectedin or trabectedin i.v. q7d × 3, respectively, at the indicated doses (see text). A representative experiment of two performed with similar results is shown. Lurbinectedin and trabectedin have similar anti-tumour efficacy. Statistical analysis: ***$P<0.001$ (two-way ANOVA; Bonferroni *post hoc* test).

and leukocytes analysed by flow cytometry. Monocytes (CD11b+ CD115+) were strongly reduced in lurbinectedin-treated mice, whereas other leukocyte populations (PMN, T and B cells) were not affected; Figure 4A shows the results obtained with the susceptible fibrosarcoma (MN/MCA1) and Figure 5A with the resistant variant (MN/MCA1-RES). The extent of monocyte reduction exceeds 50% and was totally confined to the Ly6C^{high} subset, which was reduced up to 80%. Ly6C^{high} monocytes are those predominantly recruited at peripheral inflammatory sites and at tumour tissues (Yona *et al*, 2013).Similar results were observed with trabectedin.

After three treatment cycles with lurbinectedin or trabectedin, mice were killed, and spleens and tumours analysed. Flow cytometry revealed that splenic macrophages (CD11b+ F4/80+ Ly6C^{high}) were significantly and selectively reduced (Figures 4B and 5B). Macrophages within tumours were investigated by immunohistochemical staining with F4/80. Representative pictures of treated tumours are shown in Figures 4C and 5C; the quantification analysis of different tumour sections indicated a significant reduction in the number of TAM, as well as of blood vessels (CD31) (Figures 4D and 5D). Indeed, the effect of lurbinectedin in the resistant fibrosarcoma, was slightly more significant compared with that of trabectedin (Figure 5D).

We next used a xenograft model of human ovarian cancer previously characterised (Massazza *et al*, 1989; Oliva *et al*, 2012). In line with the above mentioned results, treated tumours showed a significant decrease in the number of infiltrating macrophages and blood vessels (Supplementary Figure 3).

Overall, the results indicate that lurbinectedin *in vivo* selectively reduces the number of circulating monocytes, spleen macrophages

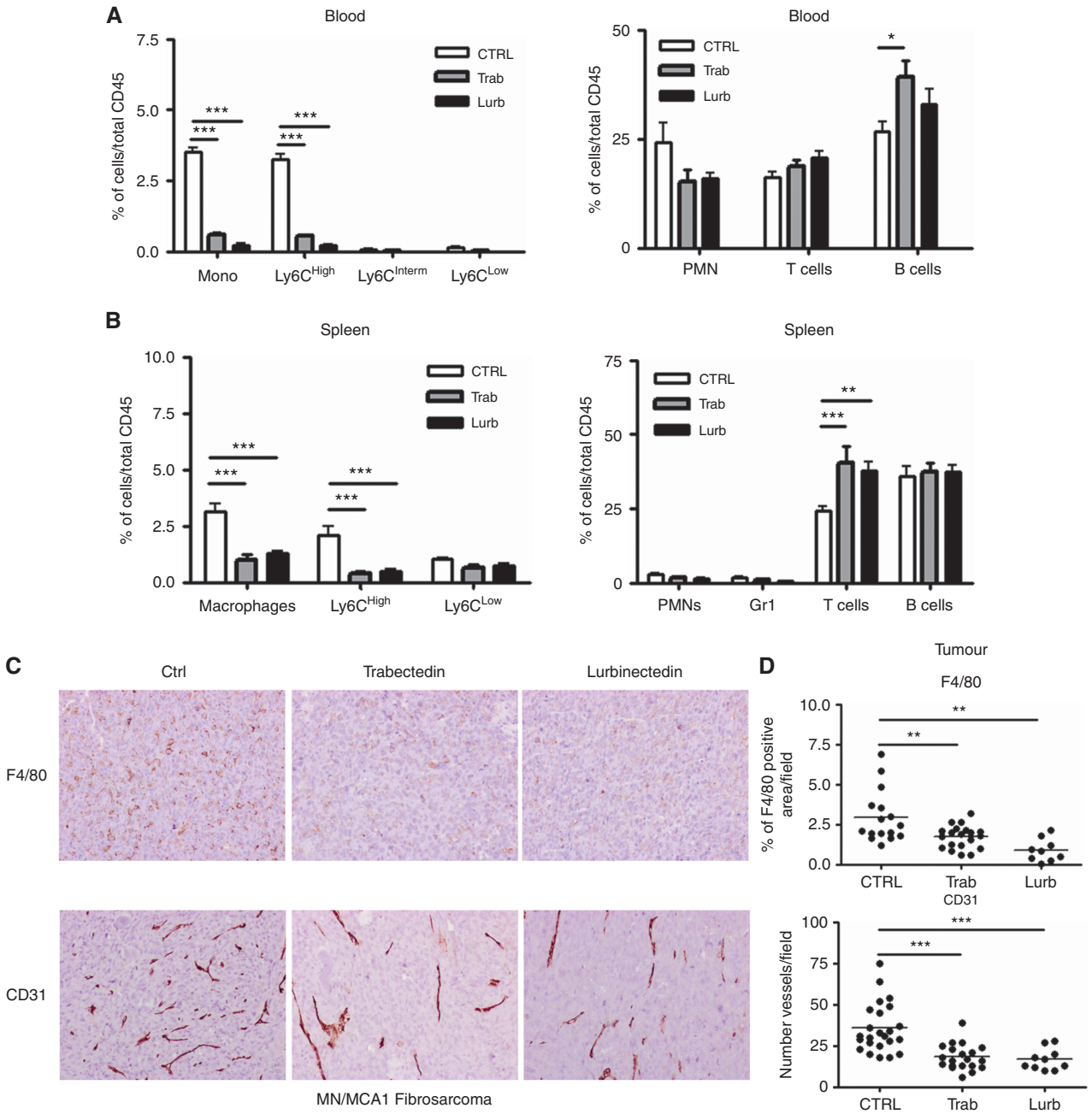

Figure 4. Effects of lurbinectedin on the tumour microenvironment of the fibrosarcoma MN/MCA1. (**A**) Flow cytometry analysis of circulating leukocytes of mice treated with lurbinectedin or trabectedin as detailed in the legend of Figure 3. Results are shown after 1 cycle of treatment. Both drugs selectively decrease the number of total monocytes (CD11b + CD115 +), especially the Ly6C^{high} monocyte subset. (**B**) Flow cytometry analysis of splenic leukocytes in mice treated after three cycles. Both drugs selectively decrease the number of macrophages (CD11b + F4/80 + Ly6C^{high}). Statistical analysis: *$P<0.05$, **$P<0.01$ and ***$P<0.001$ (two-way ANOVA; Bonferroni *T*-test. (**C**) Representative immunohistochemistry pictures of tumour sections after treatment (three cycles), stained for macrophages (F4/80) or blood vessels (CD31). (**D**) Quantification of immunohistochemistry. Both macrophages and vessels were significantly reduced in tumours treated with lurbinectedin and trabectedin. The immunoreactive areas are calculated as mean from five microscopic fields for each sample, five mice per group. Images were analysed using Image-Pro Analyzer software. Original magnification, ×20. Statistical analysis: *$P<0.05$, **$P<0.01$ and ***$P<0.001$ (Student's *t*-test).

and the density of TAM and blood vessels in the tumour microenvironment.

DISCUSSION

It is now established that macrophages within the tumour tissue fuel the buildup of an inflammatory milieu and favour disease progression (Knowles and Harris, 2007; Mantovani *et al*, 2008; Hanahan and Weinberg, 2011; Allavena and Mantovani, 2012; Coussens *et al*, 2013; De Palma and Lewis, 2013; Galluzzi *et al*, 2014; Noy and Pollard, 2014; Mantovani and Allavena, 2015; Belgiovine *et al*, 2016). Targeting of TAM for therapeutic purposes has been extensively pursued and is providing positive results in various experimental settings, either as monotherapy or combined with conventional and anti-angiogenic therapies (Zeisberger *et al*,

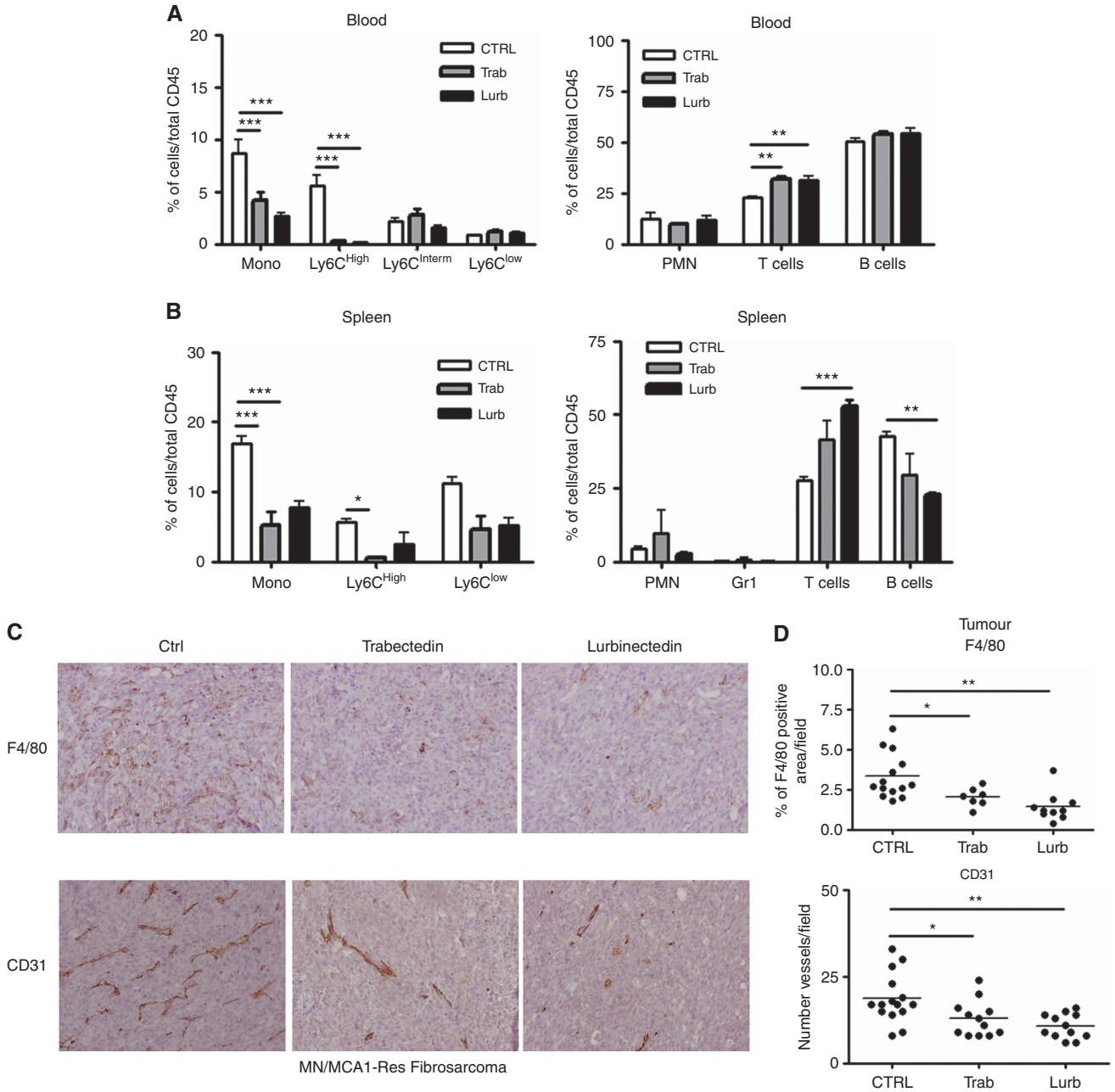

Figure 5. Effects of lurbinectedin on the tumour microenvironment of the fibrosarcoma resistant variant RES-MN/MCA1. (**A**) Flow cytometry analysis of circulating leukocytes of mice treated with lurbinectedin or trabectedin as detailed in the legend of Figure 3. Results are shown after one cycle of treatment. Both drugs selectively decrease the number of total monocytes (CD11b + CD115 +), especially the Ly6C^{high} monocyte subset. (**B**) Flow cytometry analysis of splenic leukocytes in mice treated after three cycles. Both drugs selectively decrease the number of macrophages (CD11b + F4/80 + Ly6C^{high}). Statistical analysis: *$P<0.05$, **$P<0.01$ and ***$P<0.001$ (two-way ANOVA; Bonferroni *T*-test. (**C**) Representative immunohistochemistry pictures of tumour sections after treatment (three cycles), stained for macrophages (F4/80) or blood vessels (CD31). (**D**) Quantification of immunohistochemistry. Both macrophages and vessels were significantly reduced in tumours treated with lurbinectedin and trabectedin. The immunoreactive areas are calculated as mean from five microscopic fields for each sample, five mice per group. Images were analysed using Image-Pro Analyzer software. Original magnification, ×20. Statistical analysis: *$P<0.05$, **$P<0.01$ and ***$P<0.001$ (Student's *t*-test).

2006; Zhu *et al*, 2014). Along this subject, we have previously reported that trabectedin can be considered as the prototype of a new class of anti-tumour agents with dual effects on cancer cells and on the tumour microenvironment, by specifically targeting TAM (Germano *et al*, 2010; Germano *et al*, 2013).

Lurbinectedin, an analogue of trabectedin, presents interesting clinical features with pharmacokinetic and pharmacodynamics differences compared to trabectedin (Elez *et al*, 2014; Paz-Ares *et al*, 2017; Santamaria Nunez *et al*, 2016).

In this study we have performed *in vitro* studies on human monocytes and *in vivo* experiments on macrophages from tumour-bearing mice, and demonstrated that lurbinectedin is as active as trabectedin in its selective effects on the tumour microenvironment. Mechanistically, lurbinectedin activates caspase-8-dependent apoptosis in human monocytes within few hours and at low nanomolar concentrations. We previously demonstrated that the selectivity of trabectedin for the mononuclear phagocyte lineage relies on the fact that only monocytes and macrophages express

appreciable levels of functional TRAIL-R, whereas neutrophils and T lymphocytes express high levels of the decoy non-signalling TRAIL-R3, and therefore are spared by the drug (Germano *et al*, 2013; Liguori *et al*, 2016). In treated monocytes, trabectedin upregulates the transcription of TRAIL-R and this increase appears to be sufficient to trigger caspase-8 activation and apoptosis. We also observed an increase of TRAIL-R expression in lurbinectedin-treated monocytes, indicating that a similar mechanism of monocyte apoptosis is occurring also with this analogue (not shown).

Another common effect of the two drugs is on selected inflammatory chemokines (CCL2 and CXCL8) and on VEGF, which were reduced after treatment, both in myeloid cells and in cancer cells.

The lurbinectedin-mediated effect on the tumour microenvironment was confirmed in *in vivo* experiments using a drug-resistant fibrosarcoma cell line. Even in conditions where tumour cells were totally unresponsive *in vitro* to lurbinectedin, a significant anti-tumour activity was observed, along with decreased number of circulating monocytes and TAM, and reduced tumour angiogenesis. Since at full doses the antitumour activity of trabectedin and lurbinectedin in the wild-type fibrosarcoma model was higher than that found in MN/MCA1-RES, it seems plausible to hypothesise that the drug were active by both inhibiting cancer cell growth and decreasing TAM. Comparing the two compounds for their features to impact on myeloid cells, *in vitro* and *in vivo*, we conclude that lurbinectedin and trabectedin display a very similar efficacy.

A gene expression analysis of human monocytes treated with lurbinectedin or trabectedin also indicated that similar pathways were modulated. Of particular interest are a series of genes from the Rho GTPase family that were not identified in previous analyses. RhoGTPases regulate many aspects of intracellular actin dynamics and are involved in fundamental biological functions such as signalling from integrin receptors, cell adhesion, migration and endocytosis. We confirmed that monocytes treated with lurbinectedin or trabectedin have a severe impairment in their migration activity in response to the chemokine CCL2. This finding bears importance during the active recruitment of circulating monocytes within tumours. Unlike tissue-resident macrophages, TAM originate from blood monocytes and are recalled in the TME by CCL2 and other chemotactic signals expressed by cancer or stromal cells (Noy and Pollard, 2014). Inhibition of monocyte accumulation in tumours would result in decreased TAM numbers, as observed in our *in vivo* experiments. Overall, as simplified in the scheme of Figure 6, lurbinectedin has multiple effects on the cells of the mononuclear phagocyte system: at higher doses it induces a rapid apoptosis in monocytes and TAM; at lower concentrations it affects adhesion and migration of monocytes by inhibiting the expression of genes that modulate and organise the actin cytoskeleton; at these low concentrations, lurbinectedin also inhibits the production of selected inflammatory mediators and angiogenic factors, which are important for tumour progression.

Of interest, it was recently published that the lurbinectedin-gemcitabine synergism observed in preclinical and clinical studies can be explained by the sum of the effects in both tumour cells and TAM exerted by lurbinectedin and gemcitabine (Céspedes *et al*, 2016; Paz-Ares *et al*, 2017). Indeed, TAM mediates acquired resistance of cancer cells to gemcitabine as they upregulate cytidine deaminase (CDA), one of the enzymes that catabolise gemcitabine (dFdC) in tumours into its inactive derivative dFdU (Galmarini *et al*, 2002). As a consequence, the enhanced CDA activity decreases gemcitabine intracellular levels and thus gemcitabine-induced apoptosis. Thus, the synergistic effect of the combination was explained by the effects of lurbinectedin on TAM.

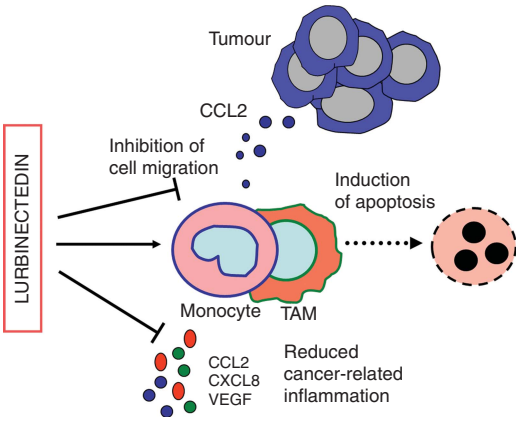

Figure 6. Schematic representation of the mechanisms of action of lurbinectedin on the tumour microenvironment. Lurbinectedin induces the apoptotic death of monocytes; at sub-cytotoxic concentrations lurbinectedin inhibits the cell migration in response to chemotactic signals (CCL2) and the production of inflammatory mediators produced in the tumour microenvironment (CCL2 and CXCL8) and the angiogenic factor VEGF.

Previous studies revealed different toxic effects for the two compounds. From the results obtained in phase I and phase II studies, it emerged that lurbinectedin maximal tolerated and recommended doses are more than three times higher than those of trabectedin (Elez *et al*, 2014; Santamaria Nunez *et al*, 2016) and plasmatic levels with an area under the curve are ~5–10 higher than that of trabectedin. As the *in vitro* cytotoxic potency of lurbinectedin towards either cancer cells or macrophages appears to be similar, it seems plausible to hypothesise that the therapeutic effect of lurbinectedin could be more marked than those of trabectedin.

Studies performed in sarcoma patients have indicated that it is possible to predict the anticancer activity of trabectedin based on the expression of DNA repair proteins. The present study suggests that other biomarkers related to tumour microenvironment should be developed to have a better prediction of patient's response.

In conclusion, our study has identified interesting mechanisms of action of lurbinectedin on the inflammatory microenvironment and selectively on the leukocyte subset of monocytes-macrophages. Macrophage targeting in tumours is now extensively pursued, but is unlikely to be successful as monotherapy. The pro-apoptotic activity of lurbinectedin on cells of the monocyte-macrophage lineage, combined with its intrinsic anti-tumour activity, makes this compound of particular interest in oncology.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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