Supplementary Materials

In vitro generation of Monocytes-Derived Macrophages (M-DM)

$CD14^+$ monocytes, were seeded in complete RPMI at the concentration of 2 million cells/ml in low adherence plates and cultured in the presence of human M-CSF (40 ng/mL, Miltenyi) for 6 days. Macrophage differentiation was evaluated by flow-cytometry. $5x10^5$ cells were stained in 0.5% FBS, HBSS solution with: anti-human CD14-FITC (clone M5E2, Bio-Legend) and anti-human CD16-PE (clone 3G8, Bio-Legend) and analyzed by a S3 Cell Sorter (Biorad). When more than 90% CD14+ cells co-express the macrophage differentiation marker CD16, M-DMs were used as following described.

M-DM gene expression analysis

To evaluate the effect of EZH2 inhibition on H3K27me3 M-DMs were treated with EPZ-6438 (10 μM, Selleckchem) for the indicated time, then core histone proteins were extracted and analyzed by western blot. Control cells were treated with the same amount of DMSO (Sigma). Due to the low stability of EPZ-6438, for treatments longer than 24 hours, cells were rechallenged with the same dose of drug or vehicle (DMSO) each 24 hours.
To evaluate the effect of EPZ-6438 on M1-polarized activation M-DMs were treated with EPZ-6438 (10 μM) for 24 hours and stimulated with LPS (100ng/ml, Enzo Life Science) during the last 4 hours. Control cells were kept in culture for the entire experimental period. In addition, cells were treated with EPZ (10μM) for 24 hours or maintained untreated for 20 hours and stimulated with LPS (100ng/ml) for the last 4 hours.
To evaluate the effect of EPZ-6438 on M2-polarized activation M-DMs were pre-treated with EPZ-6438 (10μM) for 24 hours and rechallenged with EPZ-6438 along with IL-4 (20ng/ml, Miltenyi) or IL-10 (20ng/ml, Miltenyi) for the following 18 hours. Control cells were kept in culture for the entire experimental period. Additionally, cells were treated with EPZ-6438 (10μM) for 42 hours, and cells were maintained untreated for 24 hours and stimulated with IL-4 or IL-10 (20ng/ml) for the last 18 hours.

MSTO-211H gene expression analysis

To evaluate the effect of Mo-TAMs on EMT gene expression of MSTO-211H cells, MCS were pre-treated for 48 hours with EPZ-6438 (10μM) or DMSO then, 50.000 monocytes were added to each MCS and co-cultured for additional 48 hours in presence or absence of EPZ-6438. MCS were mechanically disaggregated and tumor cells were isolated by using Miltenyi Biotech CD14 MicroBeads kit, according to the manufacturer's instructions. MSTO-211H cells, that represented the negative fraction, were analyzed for gene expression by RT-PCR.

Supplementary Table 1. Real-Time PCR primers list

Gene	Forward	Reverse
ACTB	cccaaggccaaccgcgagaagat	gtcccggccagccaggtccag
ARG1	ctgactggagacctcaagtgc	tcgtggctgtccctttgagaa
BAD	cggaggatgagtgacgagt	ccaggactggaagactcgc
BAK	aaaatggcttcggggcaag	aacgtagctgcggaaaacct
BIM	agtgggtatttctcttttga	gtgtccaattacgccgcaact
CCL2	aagatctcagtgcagaggctcg	cacagatctccttggccacaa
CCL5	gtctttgtcacccgaaag	gacaagagcaagcagaaac
CCR2B	gctggtcctgccgctg	cacacgaagcagggttttca
CCR5	ggagccctgccaaaaaatc	tgagtagagcggaggcagga
CD163	agacaaggagctgaggctagt	acagagaccgcttccatgct
CD206	cgatccgacccttccttgac	tgtctccgcttcatgccatt
CD47	tggactgagtctctgtattg	gctagagctaagatacctcaaac
CD80	tttcacttttgaccctaagc	cctgaacagaagtgagaaag
COX2	cccttgggtgtcaaaggtaa	gccctcgcttatgatctgtc
CSF1	ttaagaaggcatttctcctg	ccttgtcatgctcttcataatc
CXCL9	tcttgggcatcatcacttgct	ggtggatagtcccttggttgg
CXCL10	ggaagcactgcatcgattttg	cagaatcgaaggccatcaaga
CXCL11	gccttggctgtgatattgtgt	cactttcactgcttttacccca
CXCL12	acactccaaactgtgcccttc	ccacgtctttgccctttcatc
CSFR1	tgagcaagacctggacaagga	ggatgcaattcttggaagcg
CXCR4	aacttcagtttgttggctg	gtgtatatactgatcccctcc
E-CADHERIN	cccttcacagcagaactaac	Cacctctaaggccatctttg
HLA-A	aaaaggagggagttacactcagag	gctgtgagggacacatcagag
HLA-B	ctaccctgcggagatca	acagccaggccagcaaca
IDO	gatgtccgtaaggtcttg	cagtttgccaagacacag
IL10	ttaagggttacctgggttgccaagc	tcttggttctcagcttggggcatca
IL12B	cggtcatctgccgcaaa	tgcccattcgctccaaga
IL1B	cctactcacttaaagcccgcc	ttagaaccaaatgtggccgtg
IL6	agaacagatttgagagtagtgaggaac	ggcatttgtggttgggtcagg
LILRB1	gcttatgcttatgacctcgaac	agaacaaatctgttgtagcc
MMP2	cacagtccgccaaatgaa	cactcctgagatctgcaaac
MMP9	ccattcacgtcgtccttatg	cgctgggcttagatcattc
N-CADHERIN	gatgaaacgccgggataaa	cttcttctcctccaccttct

NOS2	aaagaccaggctgtcgttga	acgggaccggtatttcattct
NOXA	gagatgcctgggaagaagg	ttctccggaagttcagttt
P21	cagcatgacagatttctacc	cagggtatgtatgtacatgagga
PD1	cacgagggacaataggag	atagtccacagagaacacag
PDCD1	caggagggacaataggag	atagtccacagagaacacag
PD-L1	ccgaagtcatctggacaagca	tctcttggaattggtggtggt
PUMA	gacgacctcaacgcacagta	cacctaattgggctccatct
SIRPA	gaacggaacatctatattgtgg	catgcaaccttgtagaagaag
SNAIL	gatgaggacagtgggaaa	ccaaggaagagactgaagtag
TNFA	acgaacatccaaccttccca	cccaattctctttttgagccag
VEGF	tacctccaccatgccaagtg	atgattctgccctcctccttc
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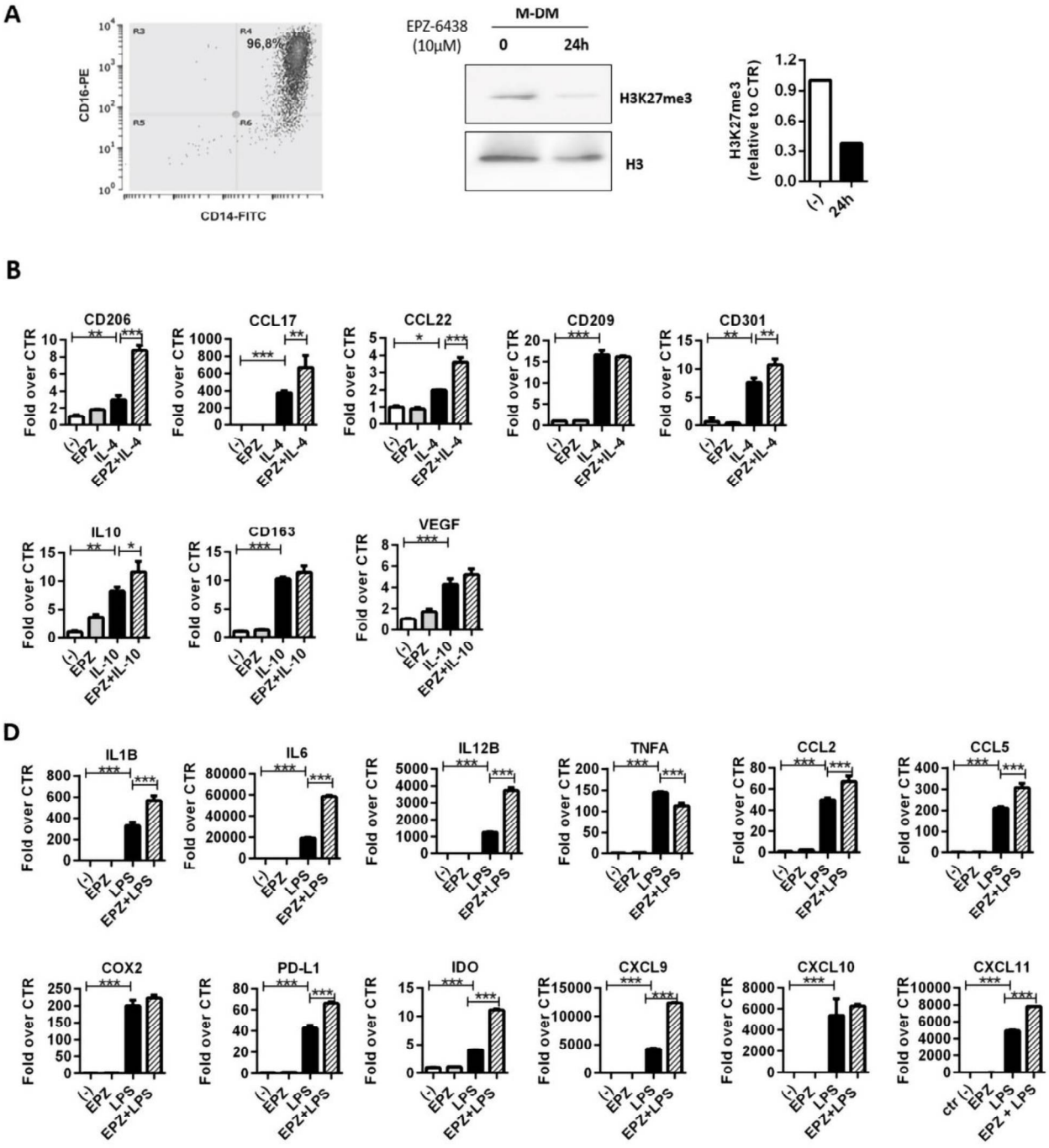

Figure S1. The effect of EZH2 inhibition affects human M-DM polarized activation. (**A**) Human monocytes cultured with 40ng/ml of MCSF for 6 days were checked for macrophage differentiation by FACS analysis of CD16 expression. Representative dot plot is shown (A, left). Human M-DM were treated with 10μM EPZ-6438 or DMSO only (-) for 24 hours, then core histone proteins were extracted and analyzed by Western blot for H3K27me3 levels. Histone H3 was used as loading control; (**B, C**) Cells were pre-treated with EPZ-6438 (10μM) for 24 hours and rechallenged with EPZ-6438 along with (**B**) IL-4 (20ng/ml) or (**C**) IL-10 (20ng/ml) for additional 18 hours. Control cells were maintained in standard medium supplemented with DMSO throughout the entire experimental period. An additional group of cells were treated with EPZ-6438 (10μM) for 42 hours and M2-polarized monocytes were cultured for 24 hours and then stimulated with (**B**) IL-4 (20ng/ml) or (**C**) IL-10 (20ng/ml) for the last 18 hours. The expression of selected (**B**) IL-4 (CD206, CCL17, CCL22, CD209 and CD301) and (**C**) IL-10 (IL10, CD163 and VEGF) inducible genes was analyzed by qRT-PCR. β-Actin was used as housekeeping gene. Normalized RT-PCR results are shown as fold increase over control monocytes (-). Data are shown as mean±SD and are representative of one out of four independent experiments with similar results. *P< 0.05, **P < 0.01 and ***P< 0.001 by two-tailed one-way ANOVA, N=3; (**D**) Monocytes were pre-treated with EPZ-6438 (10 μM) for 20 hours,

and then stimulated with LPS (100ng/ml) for the following 4 hours. Control cells were kept in culture in standard medium supplemented with DMSO for the entire experimental period. Additionally, a group of cells was treated with EPZ-6438 (10 μM) for 24 hours and a group of cells was M1-polarized by LPS (100ng/ml) stimulation during the last 4 hours. Monocytes were analyzed to evaluate the expression typical M1-inflammatory genes (TNFA, IL1B, IL6, IL12B, CCL2 and CCL5) by RT-PCR. β-Actin was used as housekeeping gene. Normalized RT-PCR results are shown as fold increase over untreated monocytes (-). Data are shown as mean±SD and are representative of one out of four independent experiments with similar results. * $P< 0.05$, ** $P< 0.01$ and *** $P< 0.001$ by two-tailed one-way ANOVA; N= 3

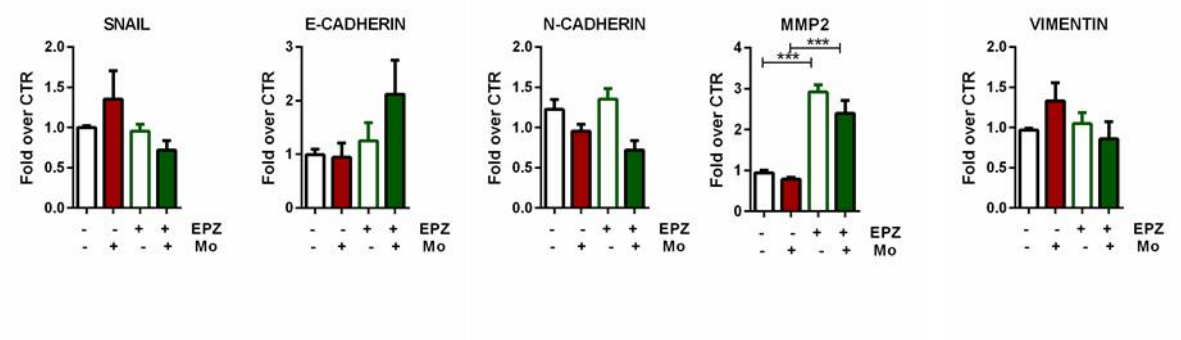

Figure S2. Mo-TAMs do not alter EMT gene expression by MSTO-211H cells. RT-PCR analysis of EMT gene expression by MSTO-211H cells derived from MCS co-cultured with monocytes. β-Actin was used as housekeeping gene. Normalized RT-PCR results are shown as fold increase over MSTO-211H cells from DMSO treated MCS (-). Data are shown as mean±SD and are representative of one out of three independent experiments with similar results. ***P< 0.01 by two-tailed one-way ANOVA, N= 3.