Supplemental figure legends

Figure S1. IC_{20} and IC_{50} determination of local anesthetics in tumor cells A-L. Human osteosarcoma U2OS cells (A-F), human cervix carcinoma HeLa cells (G-L) have been treated for 8 hours at the indicated concentrations with lidocaine (Lido), ropivacaine (Ropi), levobupivacaine (Levo), bupivacaine (Bupi), prilocaine (Prilo), chloroprocaine (Chloro). The concentration at which cell death equals 20 \% (IC_{20}) and 50 \% (IC_{50}) were determined after fitting data points with a 4-parameter log-logistic model.

Figure S2. Local anesthetics induce autophagic flux A-F. Human osteosarcoma U2OS GFP-LC3 cells were treated for 8 hours with lidocaine (Lido), ropivacaine (Ropi), levobupivacaine (Levo), bupivacaine (Bupi), prilocaine (Prilo), chloroprocaine (Chloro) at increasing concentrations, together with rapamycin (Rapa, 30 \mu M) and bafilomycin (Baf, 100 nM). Autophagy was measured by assessing the GFP-LC3 dots surface by fluorescence microscopy. Data is depicted as representative images (A, scale bar equals 10 \mu m) and bar charts (B) and expressed as the mean ± SD of one experiment representative of three independent experiments. U2OS RFP-FYVE cells were treated with Lido (4 mM), Ropi (2 mM), Levo (1 mM), Bupi, (1 mM), Prilo (4 mM), Chloro (3 mM) or thapsigargin (TG, 5 \mu M). Data is depicted as representative images (C, scale bar equals 10 \mu m) and bar charts (D) and expressed as the mean ± SEM of three independent experiments. Human osteosarcoma U2OS mCherry-GFP-LC3 cells were treated with Lido (4 mM), Ropi (2 mM), Levo (1 mM) or Bupi (1 mM). Autophagic flux was measured by assessing the shift of GFP and mCherry-LC3 dots surface by fluorescence microscopy. Data is depicted as representative images (E, scale bar equals 10 \mu m) and line plots (F, grey line depicts negative control and rapamycin and the black line indicates bafilomycin and rapamycin + bafilomycin) (n=1 representative of three independent experiments).

Data information: (B) Statistics were calculated using pairwise multiple comparison with a Benjamin-Hochberg correction. Stars indicate the p-value of each treatment compared with untreated condition (black stars) or compared with bafilomycin condition (grey stars). (D) Data were normalized to untreated condition. Samples were compared with Student’s t test. *p<0.05, **p<0.01, ***p<0.001

Figure S3. Local anesthetics fail to induce cancer cell death in Atg5\(^{-}\) cells A. U2OS cells stably expressing GFP-LC3 and U2OS GFP-LC3 knock-out for Atg5 (U2OS GFP-LC3
were treated for 6 hours with lidocaine (Lido, 4 mM), ropivacaine (Ropi, 2 mM), levobupivacaine (Levo, 1 mM), bupivacaine (Bupi, 1 mM), prilocaine (Prilo, 4 mM), chloroprocaine (Chloro, 3 mM), tunicamycin (TM, 5 µM), thapsigargin (TG, 5 µM), rapamycin (Rapa, 30 µM). Healthy number cells are depicted. (n=3)

Data information: data were normalized to untreated condition for each cell line. Statistics were calculated using pairwise multiple comparison with a Benjamin-Hochberg correction. Stars indicate the p-value of each treatment compared with untreated condition. Hashes indicate p-value of knock-out cells compared with the wild-type cells for the same treatment.

* p<0.05, ** p<0.01, *** p<0.001

Figure S4. Immunoblot of U2OS GFP-LC3 EIF2A kinases 1 to 4 knock-out, MCA205 EIF2A kinase 3 knock-out and MCA205 ATG5

Figure S5. Local anesthetics induce all arms of the endoplasmic reticulum stress response A-F. U2OS cells stably expressing pSMALB-ATF4.5rep (detecting alternating usage of the open reading frame of ATF4), GFP-ATF6 (detecting ATF6 translocation from the cytoplasm to the nucleus), XBP1ΔDBD-venus (detecting alternative splicing of XBP1 mRNA) were treated for 12 hours, 6 hours and 12 hours respectively with lidocaine (Lido, 4 mM), ropivacaine (Ropi, 2 mM), levobupivacaine (Levo, 1 mM), bupivacaine (Bupi, 1 mM), prilocaine (Prilo, 4 mM), chloroprocaine (Chloro, 3 mM) and tunicamycin (TM, 5 µM). pSMALB-ATF4.5rep was assessed by fluorescence microscopy and the average nuclear intensity is represented (A, B). XBP1s abundance was measured by assessing fluorescence intensity (C, D). ATF6 activation was measured as an increase in the ratio of the nuclear to cytoplasmic fluorescence intensity (E, F). Data are represented as the mean ± SEM of three independent experiments. Data were normalized to untreated condition. Data are depicted as representative images (scale bar 10 µm) and bar charts. Samples were compared with Student’s t test. *p<0.05, **p<0.01, ***p<0.001

Figure S6. Local anesthetics promote antitumor immune responses in vivo A-B. Murine fibrosarcoma MCA205 cells were injected subcutaneously into the flank of immunocompetent C57Bl/6 mice with 7 mice per group (n= 6 for lidocaine (Lido),
bupivacaine (Bupi) and prilocaine (Prilo)) (A) and of immunodeficient nu/nu mice (B) with 8 mice per group. When tumors became palpable, mice were treated for 2 days by intratumoral injections of PBS as control (PBS) or lidocaine (Lido, 3 mg/kg) or levobupivacaine (Levo, 2 mg/kg) or bupivacaine (Bupi, 1 mg/kg) or prilocaine (Prilo, 2 mg/kg) or chloroprocaine (Chloro, 2.5 mg/kg). Mean tumor sizes were compared using a type II ANOVA (Analysis of Variance) test and pairwise Wilcoxon test. Data are represented as boxplots. Overall survival was compared with a log-rank test. *p<0.05, **p<0.01, ***p<0.001

Figure S7. IC$_{20}$ and IC$_{50}$ determination of lidocaine and ropivacaine in tumor cells lacking EIF2AK3 or ATG5 A-C. Human osteosarcoma U2OS cells wild-type (A), U2OS EIF2AK3$^{-/-}$ (B) and U2OS Atg5$^{-/-}$ (C) have been treated for 8 hours at the indicated concentrations with lidocaine or ropivacaine. The concentration at which cell death equals 20 % (IC$_{20}$) or 50 % (IC$_{50}$) was determined after fitting data points with a 4-parameter log-logistic model.

Figure S8. Cell death mechanisms induced by local anesthetics A-D. Human osteosarcoma U2OS cells EIF2AK3$^{-/-}$ (A) and U2OS cells knock-down for Atg5 (B) have been treated for 8 hours with staurosporine (STS 1 µM), lidocaine (Lido, 4 mM) or ropivacaine (Ropi, 2 mM). Human osteosarcoma U2OS wild-type cells and U2OS Atg5$^{KD}$ cells were validated by immunoblot (C). Human osteosarcoma U2OS wild-type cells have been treated for 8 hours with staurosporine (STS 1 µM), lidocaine (Lido, 4 mM), TNF-alpha (T, 0.02 µg/ml), z-VAD-fmk (Z, 50 µM), Smac-BV6 (B, 10 µM), erastin (E, 10 µM), ferrostatin (F, 10 µM) or necrostatin (N, 10 µM) (D). Cell death modalities were determined by DIOC and DAPI staining and flow cytometry. Data are expressed as the percentage of DIOC negative, DAPI negative (DIOC$^{-}$ DAPI$^{-}$), and DAPI positive (DAPI$^{+}$). Data are represented as the mean ± SEM of three independent experiments (A and D) or four independent experiments (B).

Data information: data were normalized to untreated condition for each cell line. Statistics were calculated using pairwise multiple comparison with a Benjamin-Hochberg correction. Stars indicate the $p$-value of each treatment compared with untreated condition for each cell line (black stars = DAPI$^{+}$ comparison; grey stars = DIOC$^{-}$ DAPI$^{-}$ comparison). Hashes indicate $p$-value of knock-out cells or knock-down cells compared with the wild-type cells for the same treatment (black hashes = DAPI$^{+}$ comparison; grey hashes = DIOC$^{-}$ DAPI$^{-}$).

*:*$p<0.05$, **:**$p<0.01$, ***:**:**$p<0.001$
Figure S9. Local anesthetics fail to promote anti-tumor response in tumors expressing an ecto-ATPase CD39 A-D. Murine fibrosarcoma MCA205 wild-type cells and MCA205 CD39 cells were validated by immunoblot (A) and flow cytometry (B). MCA205wt and MCA205 CD39 cells were injected s.c. into the flank of immunocompetent C57Bl/6 mice with 8 mice per group (C, D). When tumors became palpable, mice were treated for 2 days by intratumoral (i.t.) injections of PBS as control (PBS) or ropivacaine (Ropi, 4 mg/kg). Mean tumor size was compared using a type II ANOVA (Analysis of Variance) test and pairwise Wilcoxon test. Data are represented as boxplots. Overall survival was compared with a log-rank test. *p<0.05, **p<0.01, ***p<0.001.

Figure S10. Local anesthetics promote the engulfment of tumor cells by bone marrow-derived dendritic cells A-D. Murine fibrosarcoma MCA205 cells were stained with CellTracker Orange (CMTMR+) and treated for 24 hours with lidocaine (Lido, 6 mM), ropivacaine (Ropi, 4 mM) or oxaliplatin (Oxa, 500 µM). Then, cells were co-cultured with differentiated bone marrow-derived dendritic cells for 4 hours at 37°C. Dendritic cells (DCs) were determined by immunostaining using a CD11c-specific antibody (CD11c-APC). CMTMR+ and CD11c+ were assessed by cytometry flux. Data are depicted as one representative of two independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Figure S11. Local anesthetics do not inhibit the transcription A-C. Human osteosarcoma U2OS wild-type cells were treated with lidocaine (Lido, 4 mM), ropivacaine (Ropi, 2 mM), levobupivacaine (Levo, 1 mM), bupivacaine (Bupi, 1 mM), prilocaine (Prilo, 4 mM), chloroprocaine (Chloro, 3 mM), dactinomycin (Dact, 1 µM) for 2.5 hours and a treatment pursued in the presence of 1 mM 5-ethynyl uridine (EU). Then, EU was stained with an Alexa Fluor®-488-coupled azide. Data is depicted as representative images (scale bar equals 10 µm) and bar chart. Data are represented as the mean ± SEM of three independent experiments. Samples were compared with Student’s t test. *p<0.05, **p<0.01, ***p<0.001.

Figure S12. Mitochondrial uncouplers induce autophagy and all arms of endoplasmic reticulum stress A-H. U2OS cells stably expressing pSMALB-ATF4.5rep (detecting alternating usage of the open reading frame of ATF4), GFP-ATF6 (detecting ATF6 translocation from the cytoplasm to the nucleus), XBP1ΔDBD-venus-RFP-FYVE (detecting alternative splicing of XBP1 mRNA and FYVE activation) were treated with respiratory chain complex I inhibitor BAY87-2243 (B87, 50 µM), carbonyl cyanide m-chloro-phenyl
hydrazone (CCCP, 50 µM), 2,4-Dinitrophenol (DNP, 50 µM), rotenone (Rot, 50 µM), rapamycin (Rapa, 30 µM) or tunicamycin (TM, 5 µM) for 12 hours, 6 hours and 12 hours respectively. Autophagy was measured by assessing the RFP-FYVE dots surface by fluorescence microscopy (A, B). pSMALB-ATF4.5rep was assessed by fluorescence microscopy and the average intensity of pSMALB-ATF4.5rep is represented (C, D). XBP1s abundance was measured by assessing fluorescence intensity (E, F). ATF6 activation was measured as an increase in the ratio of the nuclear to cytoplasmic fluorescence intensity (G, H). Data were normalized to untreated condition. Data are represented as the mean ± SEM of three independent experiments. Data is depicted as representative images (scale bar equals 10 µm) and bar charts. Data were compared with Student’s t test. *p<0.05, **p<0.01, ***p<0.001

**Figure S13. Mitochondrial uncouplers induce hallmarks of immunogenic cell death A-C.** Human osteosarcoma U2OS wild-type cells and U2OS cells stably expressing HMGB1-GFP were treated for 24 hours (A, B) or 6 hours (C) with respiratory chain complex I inhibitor BAY87-2243 (B87, 50 µM), carbonyl cyanide m-chloro-phenyl hydrazone (CCCP, 50 µM), 2,4-Dinitrophenol (DNP, 50 µM), rotenone (Rot, 50 µM), mitoxantrone (MTX, 3 µM). ATP release was assessed by bioluminescence (A) HMGB1 release was assessed by measuring GFP fluorescence decrease by fluorescence microscopy (B). Calreticulin (CALR) exposure was determined by immunofluorescence staining and flow cytometry. Data are expressed as the percentage of CALR positive, DAPI negative (CALR⁺ DAPI⁻) cells (C). Data are represented as the mean ± SD of one representative of three independent experiments. Samples were compared with Student’s t test. *p<0.05, **p<0.01, ***p<0.001.

**Figure S14. Mitochondrial uncouplers stimulate an anti-tumor response potentiated by immunotherapy in vivo A-F.** Murine fibrosarcoma MCA205 cells were injected subcutaneously (s.c.) into the flank of immunocompetent C57Bl/6 mice with 7 mice per group (A) or 9 mice per group (B) (n= 8 for PBS and FCCP+anti-PD-1 (B)) (A, B). Murine breast cancer E0771 cells were injected s.c. into the flank of immunocompetent C57Bl/6 mice with 8 mice per group (n=7 for FCCP + anti-PD-1 (D)) (C, D). Murine colon adenocarcinoma MC38 cells were injected s.c. into the flank of immunocompetent C57Bl/6 mice with 8 mice per group (n=7 for CCCP+ anti-PD-1 (E) and FCCP (F)) (E, F). When tumors became palpable, mice were treated for 2 days by intratumoral injection of PBS as control (PBS) or carbonyl cyanide m-chloro-phenyl hydrazone (CCCP, 0.25 mg/kg) or
p-trifluoromethoxy-carbonyl-cyanide-phenyl hydrazone (FCCP, 0.25 mg/kg). Anti-PD-1 intraperitoneal injection were performed at days 8, 12 and 16 after injection of local anesthetics.

Data information: Figures C and D provide from the same experiment. PBS and anti-PD-1 groups are the same in both figures. Figures E and F provide from the same experiment. PBS and anti-PD-1 groups are the same in both figures. Mean tumor sizes were compared using a type II ANOVA (Analysis of Variance) test and pairwise Wilcoxon test. Data are represented as boxplots. Overall survival was compared with a log-rank test. *p<0.05, **p<0.01, ***p<0.001, compared with controlled mice, †p<0.05, ‡p<0.01, §§p<0.001, compared with anti-PD-1-single-treated mice and ††p<0.05, ‡‡p<0.01, §§§p<0.001, compared with mitochondrial uncoupler-single-treated mice.

Table S1. Prospective clinical trials evaluating the effects of local anesthetics on cancer.

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<th>ICU</th>
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<th>NA</th>
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<th>PCS/MCS Physical and Mental Health Composite Scores</th>
<th>PONV</th>
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