Macrophages Promote Growth of Squamous Cancer Independent of T-cells

F.L. Wu, K. Nolan, A.A. Strait, L. Bian, K.A. Nguyen, J.H. Wang, A. Jimeno, H.M. Zhou, C.D. Young, and X.J. Wang

Appendix

Appendix Materials and Methods

Flow cytometry and mass cytometry (CyTOF)

Excised tumors were minced into small pieces and digested in Hank's Balanced Salt Solution (HBBS) (Gibco, Grand Island, NY, USA) containing type II collagenase at 37 °C for 40 minutes. To obtain splenocytes, spleens were minced and filtered through a 70 μm mesh filter and adjusted to 1 million cells/mL in PBS with 3.0% fetal bovine serum (FBS) (Gibco). RBC lysis buffer (Invitrogen, Carlsbad, CA, USA) was used to remove red blood cells from tumor cell suspension followed by staining of dead cells with Cell-ID Cisplatin (Fluidigm, San Francisco, CA, USA). Cells were washed with Maxpar Cell Staining Buffer (Fluidigm), resuspended in Fix I buffer (Fluidigm), washed with Maxpar Cell Staining Buffer again and incubated with cell surface staining antibodies CD45 (30-F11), CD8a (53-6.7) and F4/80 (BM8-Biolegend) to label tumor-associated leukocytes (unless otherwise stated, antibodies were purchased from BD Biosciences, San Jose, CA, USA). Cells were washed twice with Maxpar Cell Staining Buffer. FITC-F4/80 (Biolegend, San Diego, CA, USA) and propidium iodide (Sigma-Aldrich, St Louis, MO, USA) were also used. Prior to CyTOF data acquisition on a Helios Mass Cytometer, cells were stained with antibodies listed in appendix table 1 and cell concentration was adjusted to 2.5-5 X 10^5/mL with Maxpar Water and filtered through 30 micron mesh strainers.

Immunostaining, TUNEL assay and western blot

The following antibodies were used for immunostaining: F4/80, FLIP, pSmad1/5/8 and E-cadherein (Cell Signaling Technologies, Danvers, MA, USA); CD31 (BD Bioscience); VEGFA, pBcl-2, Bcl-xL and NFκB p-p65 (Invitrogen); N-cadherin, pSmad3, and TNFα (Abcam, Cambridge, MA); Keratin 5 and Ki-67 (eBioscience, San Diego, CA, USA). Secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen) were used for immunofluorescence and secondary antibodies conjugated to horseradish peroxidase were used in conjunction with DAB (brown) and AEC (red) chromagen detection. Slides were counterstained with DAPI, propidium iodide or hematoxylin. The number of $F4/80^+$ cells were counted in 8-10 fields of view, normalized to area and averaged across 4-8 different samples. Vessel area was determined by quantifying lumens surrounded by $CD31^+$ cells and normalized to total area.

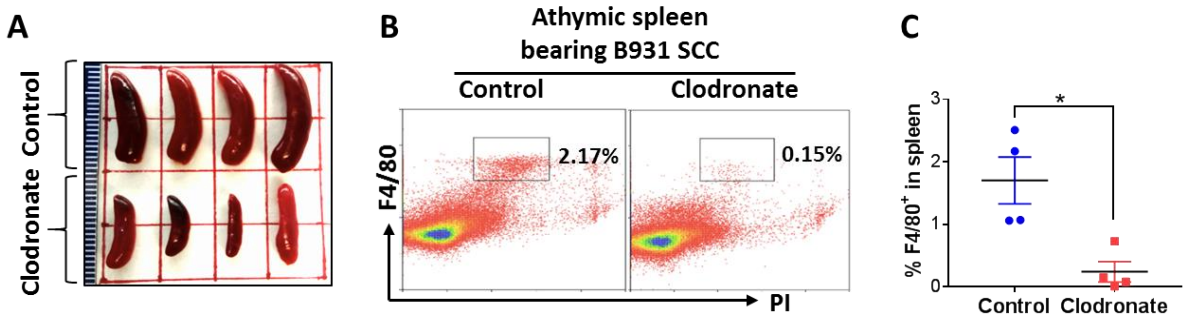
Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay kit (Promega, Madison, WI, USA) was used to detect apoptotic cells according to the manufacturer's instructions. Apoptotic cells were examined at 200x magnification in at least 10 fields in each tumor and quantified as the number of apoptotic cells/mm^2 ± SEM. At least 5 tumors/group were used for quantification.

The following antibodies were used in western blot analyses: pSmad3, FLIP, cleaved caspase-8, Caspase-3 and Tubulin (Cell Signaling Technologies), VEGFA, pBcl2, and Bcl-xL (Invitrogen), GAPDH or β-actin (Santa Cruz Biotechnologies, Dallas, Texas, USA). Horseradish peroxidase conjugated secondary antibody was used to detect primary antibodies. Signal was detected by enhanced chemiluminescence and exposure of X-ray film.

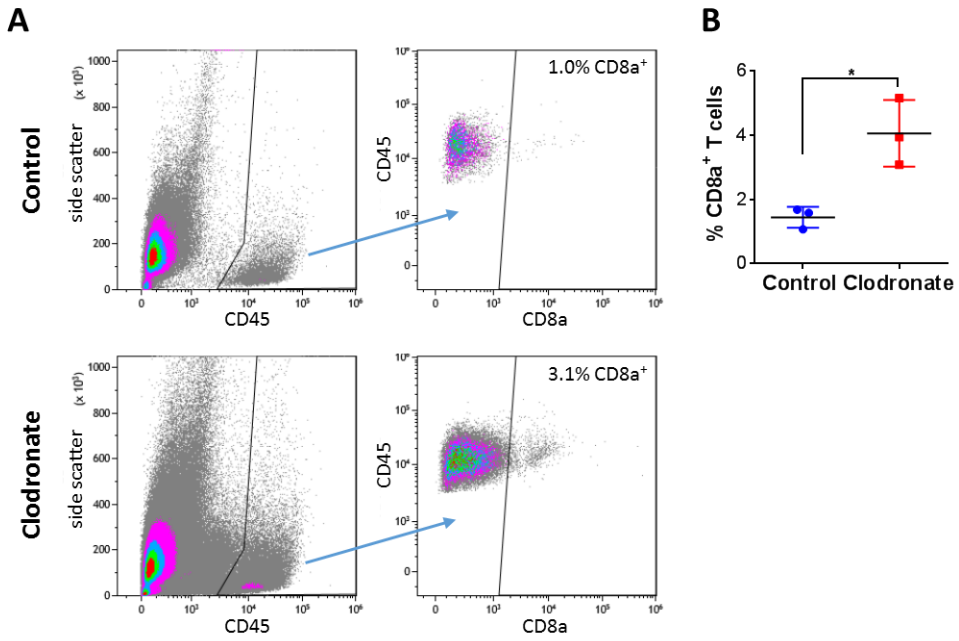
Appendix Table 1: Antibodies used in mass cytometry

Target	Clone	Ion
CD11b	M1/70	148Nd
CD11c	N418	142Nd
NK1.1	PK136	170Er
CD3e	145-2C11	152Sm
CD4	RM4-5	172Yb
CD45	30-F11	89Y
CD8a	53-6.7	168Er
F4/80	BM8	146Nd
Ly6C	HK1.4	150Nd
Ly6G	1A8	141Pr
Arg1	Polyclonal	166Er
iNos	CXNFT	161Dy

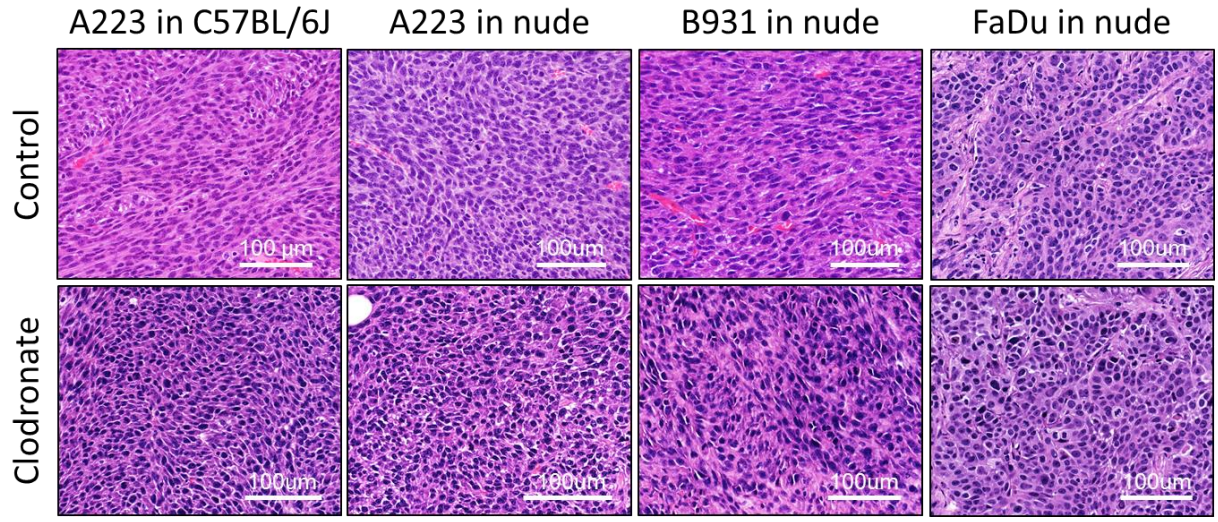
Method: cells were stained following manufacturer instructions for intracellular staining (Fluidigm). Live cells were identified by cisplatin-negative staining, and nucleated cells were identified by iridium-positive DNA staining.



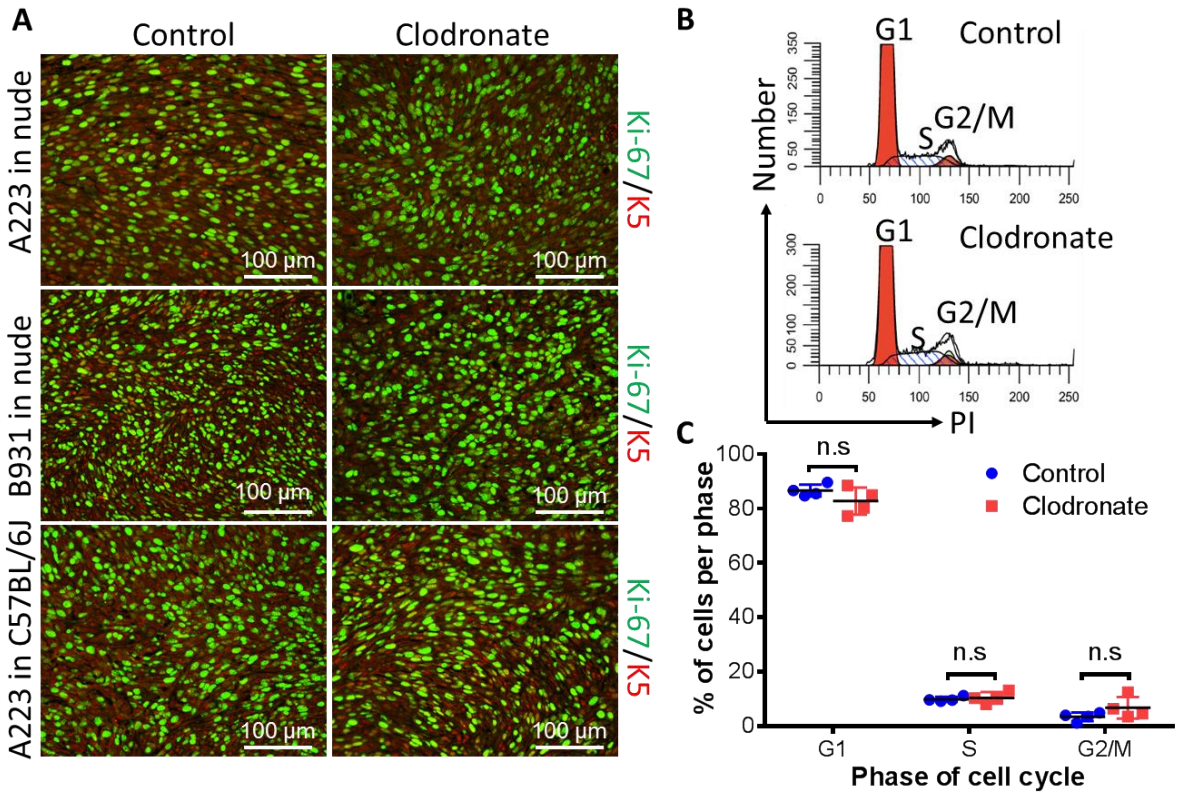
Appendix Figure 1: Clodronate treatment reduces spleen size and splenic macrophages. B931 tumor-bearing athymic nude mice were treated with clodronate liposomes and control mice were treated with empty liposomes. Spleens were harvested, photographed (A), dissociated into single cells for flow cytometry analysis of F4/80$^+$ cells (B), quantified and analyzed by Student's *t* test (C). Values are presented as mean ± SEM. **P* < 0.05.



Appendix Figure 2: Clodronate treatment increases the number of CD8a$^+$ T cells in tumors. A223 cells grown in C57BL/6J mice treated with control or clodronate liposomes were harvested, digested into single cell suspension, stained with antibodies against CD45 and CD8a and analyzed by flow cytometry to determine the number of CD8a$^+$ T cells per tumor. **A.** Representative flow cytometry gating plots from a control and a clodronate tumor to determine the percentage of CD45$^+$ cells that were also CD8a$^+$. **B.** Quantification of three tumors in each group, Student's *t* test, values are presented as mean ± SEM. *$P < 0.05$.

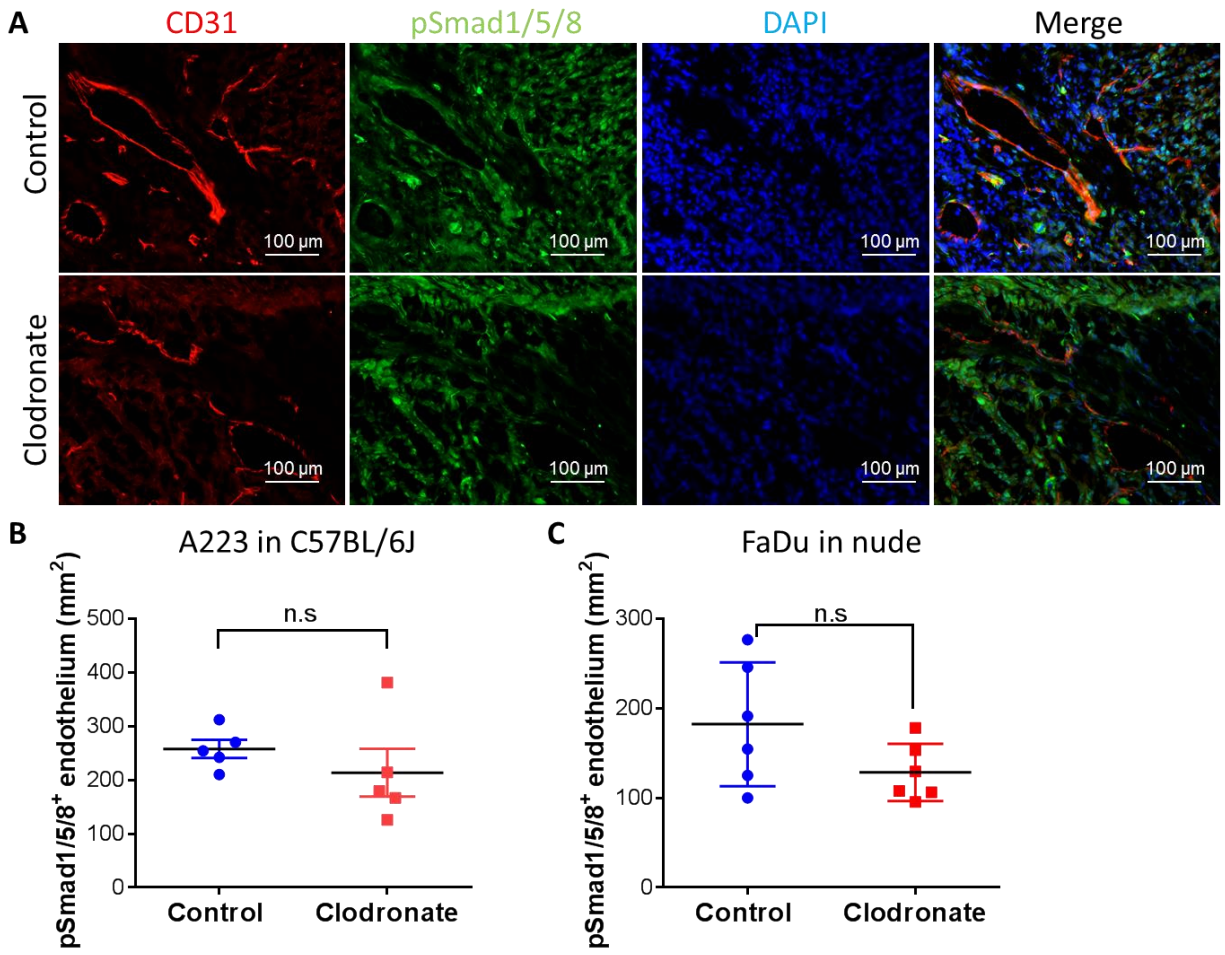


Appendix Figure 3: Clodronate treatment does not alter overall tumor histology. Mice bearing A223, B931 or FaDu tumors were treated with clodronate liposomes or empty liposomes (control). Tumors were harvested, fixed, embedded, sectioned and stained with hematoxylin and eosin. Images are representative of each tumor and treatment group.



Appendix Figure 4: Clodronate treatment does not affect tumor cell proliferation.

A. SCC cell lines grown in the indicated mouse strains treated with control or clodronate liposomes were harvested and stained for Ki-67 (green), K5 (red), DAPI (blue). **B-C.** A223 cells grown in C57BL/6J mice treated with control or clodronate liposomes were harvested, digested into single cell suspension, stained with propidium iodide (PI) and analyzed by flow cytometry to determine the cell cycle phase. **B.** Raw flow cytometry data for a single representative tumor in each group. **C.** Quantification of four tumors in each group, Student's *t* test, values are presented as mean ± SEM. n.s, no significance.



Appendix Figure 5: Clodronate treatment does not alter endothelial pSmad1/5/8.

A-B. A223 tumors in C57BL/6J mice treated with control or clodronate liposomes were harvested and stained for pSmad1/5/8 (green), CD31 (red), DAPI (blue). Representative photomicrographs are presented in panel A and quantified in panel B (Statistics by Student's t test; n.s, not significant).

C. FaDu tumors in athymic nude mice were treated, harvested, stained and quantified as explained in panels A and B.