Immunity

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CXCR1 and CXCR2 Chemokine Receptor Agonists Produced by Tumors Induce Neutrophil Extracellular Traps that Interfere with Immune Cytotoxicity

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Neutrophils and Cancer

- Pro-tumorigenic
  - Pro-angiogenic
  - Immunosuppressive
- Presence of tumor-infiltrating neutrophils = poor prognosis
- **G**ranulocytic **M**yeloid-**D**erived **S**uppressor **C**ells (GR-MDSCs)
  - Subset of neutrophil-like cells
  - T-cell suppressive functions
  - Expanded in cancer-bearing hosts

CXCR1 and CXCR2

- Expressed on Neutrophils & GR-MDSCs
- CXCR1 → CXCL6 and CXCL8
- CXCR2 → CXCL1 – CXCL7 chemokines; sharing ELR motif
- ELR^+ CXCL chemokines recruit myeloid cells to tumors (mostly Neutros)

- CXCL1, CXCL2, CXCL8 produced by cancer cells
- CXCR1 / CXCR2 inhibition = promoted T cell response against tumors due to limited GR-MDSC or Neutrophil infiltration

Do Neutrophil Chemoattractants induce NETosis in Neutrophils and GR-MDSCs?

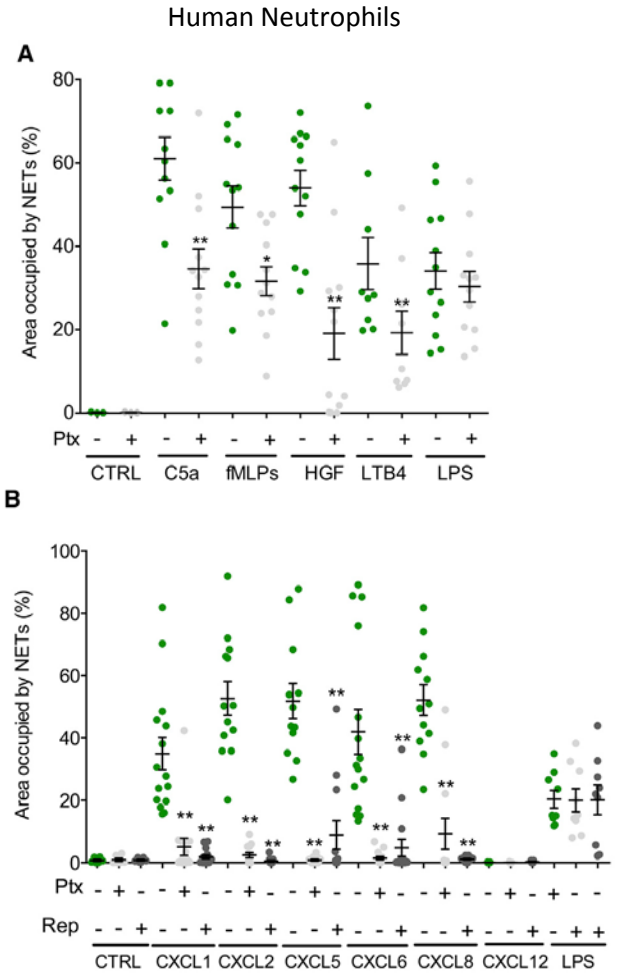
Treatment of

- Healthy donor Neutrophils
- GR-MDSCs from cancer patients
- Mouse GR-MDSCs from mice bearing 4T1 tumors

with

- Chemoattractants
- +/- Pertussis Toxin (Ptx) as G_i subunit inhibitor
- +/- Reparixin (Rep) as specific allosteric CXCR1 and CXCR2 inhibitor

Fig.1 Neutrophil Chemoattractants induce NETosis in Neutrophils and GR-MDSCs

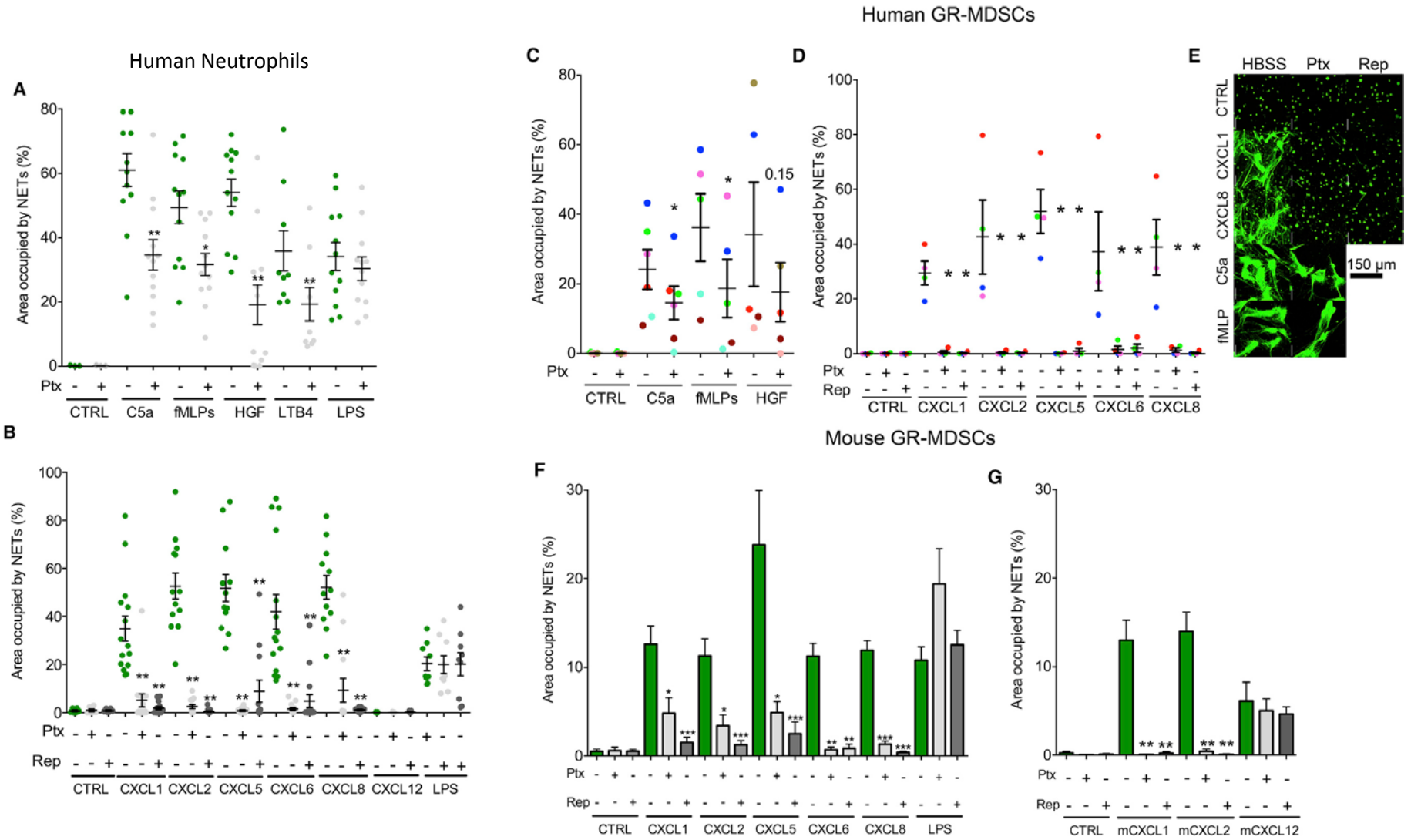


a. Reduced NETosis upon G_i subunit inhibition (PTX)

b. Chemokine-induced NETosis acting via CXCR1 / CXCR2 → reduced upon PTX treatment

   Reparixin reduced CXCR1 / CXCR2 dependent NET induction

Fig.1 Neutrophil Chemoattractants induce NETosis in Neutrophils and GR-MDSCs



a. Reduced NETosis upon G_i subunit inhibition (PTX)

b. Chemokine-induced NETosis acting via CXCR1 / CXCR2 → reduced upon PTX treatment

   Reparixin reduced CXCR1 / CXCR2 dependent NET induction

c. similar results in GR-MDSCs
d. from cancer patients as seen
e. in Neutrophils

f. Mouse GR-MDSCs from 4T1
g. tumor bearing mice showed similar activation pattern as human Neutros or GR-MDSCs

Do Tumor-derived Factors Induce NETosis in Neutrophils and GR-MDSCs by Activating CXCR1 and CXCR2 Receptors?

Treatment of

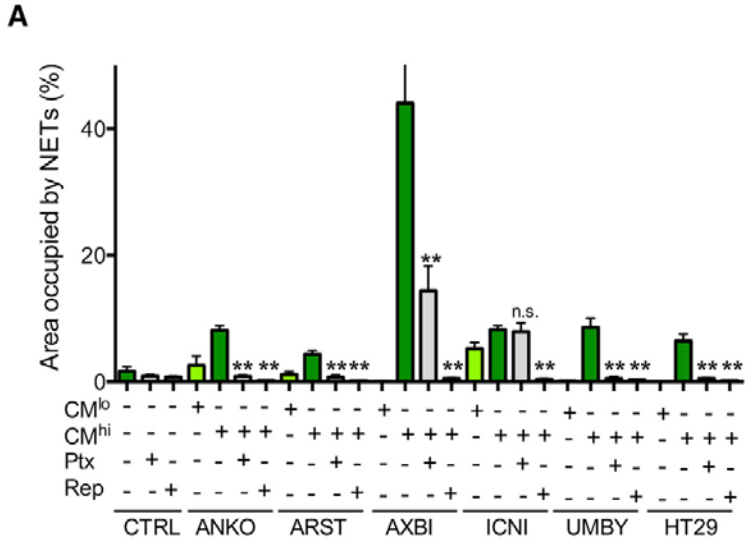
- Healthy donor Neutrophils
- GR-MDSCs from cancer patients

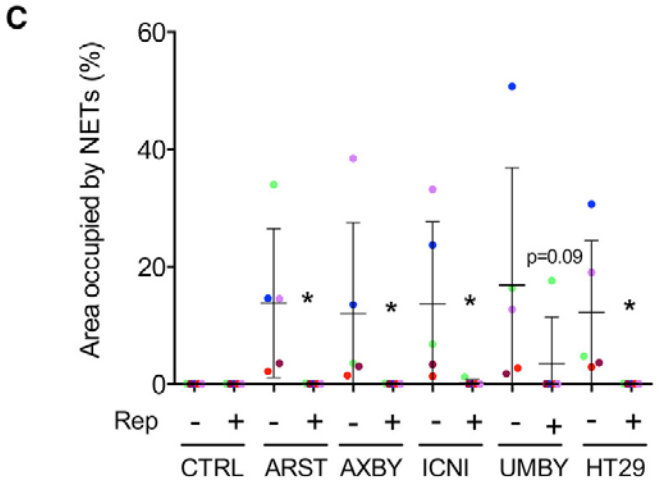
with

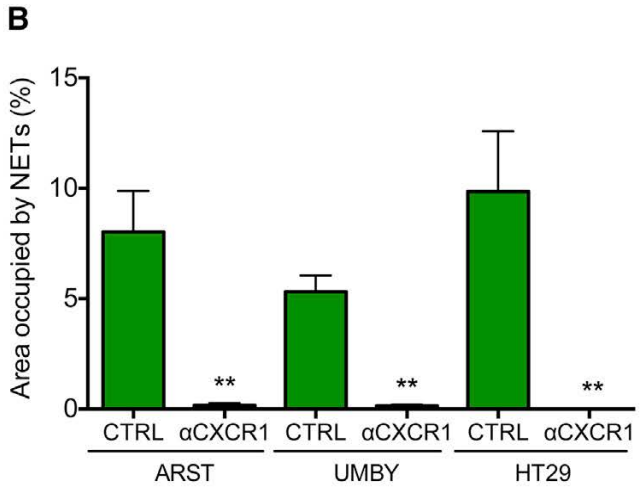
- Culture supernatant of
  five different primary melanoma cell lines and
  one colon carcinoma cell line (HT29)

Generation of 3D tumor spheroids with Neutrophil co-culture

Fig.2 Tumor-derived Factors Induce NETosis in Neutrophils and GR-MDSCs by Activating CXCR1 and CXCR2 Receptors





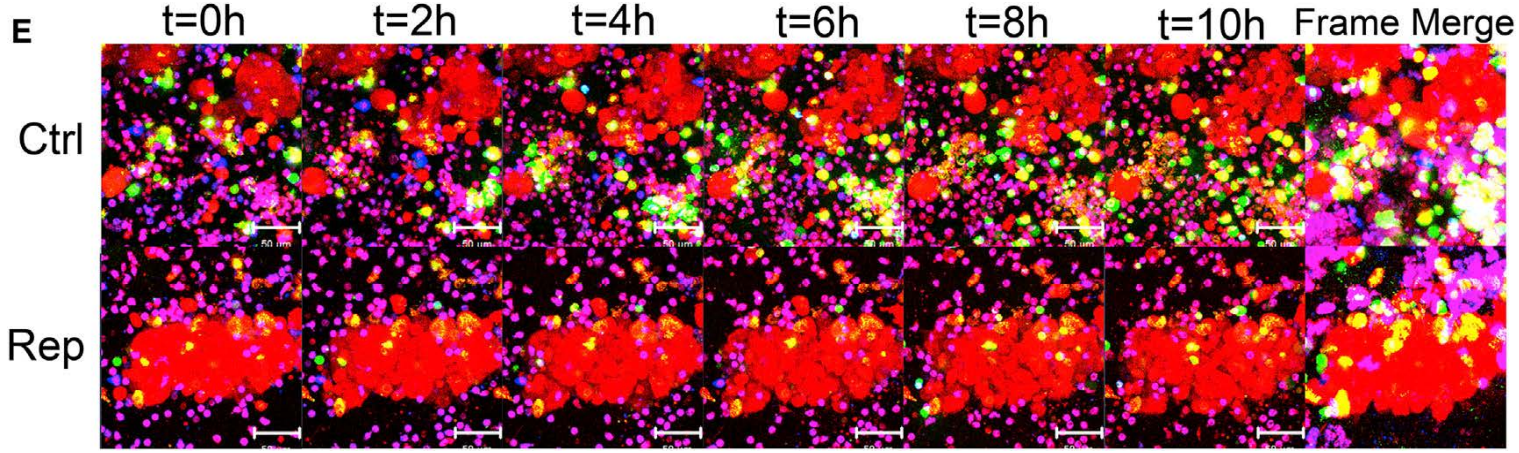


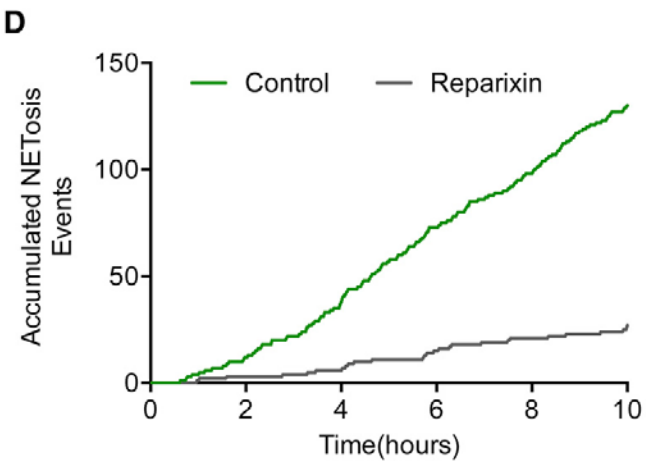
a. CM^{hi} = potent NET inducer (independent of cancer cell line)
both, Ptx and Rep = potent inhibitors

b. Anti-CXCR1 antibody could abolish NET-inducing capacity of cancer cell line CM^{hi}

c. similar results in GR-MDSCs upon Reparixin induced CXCR inhibition

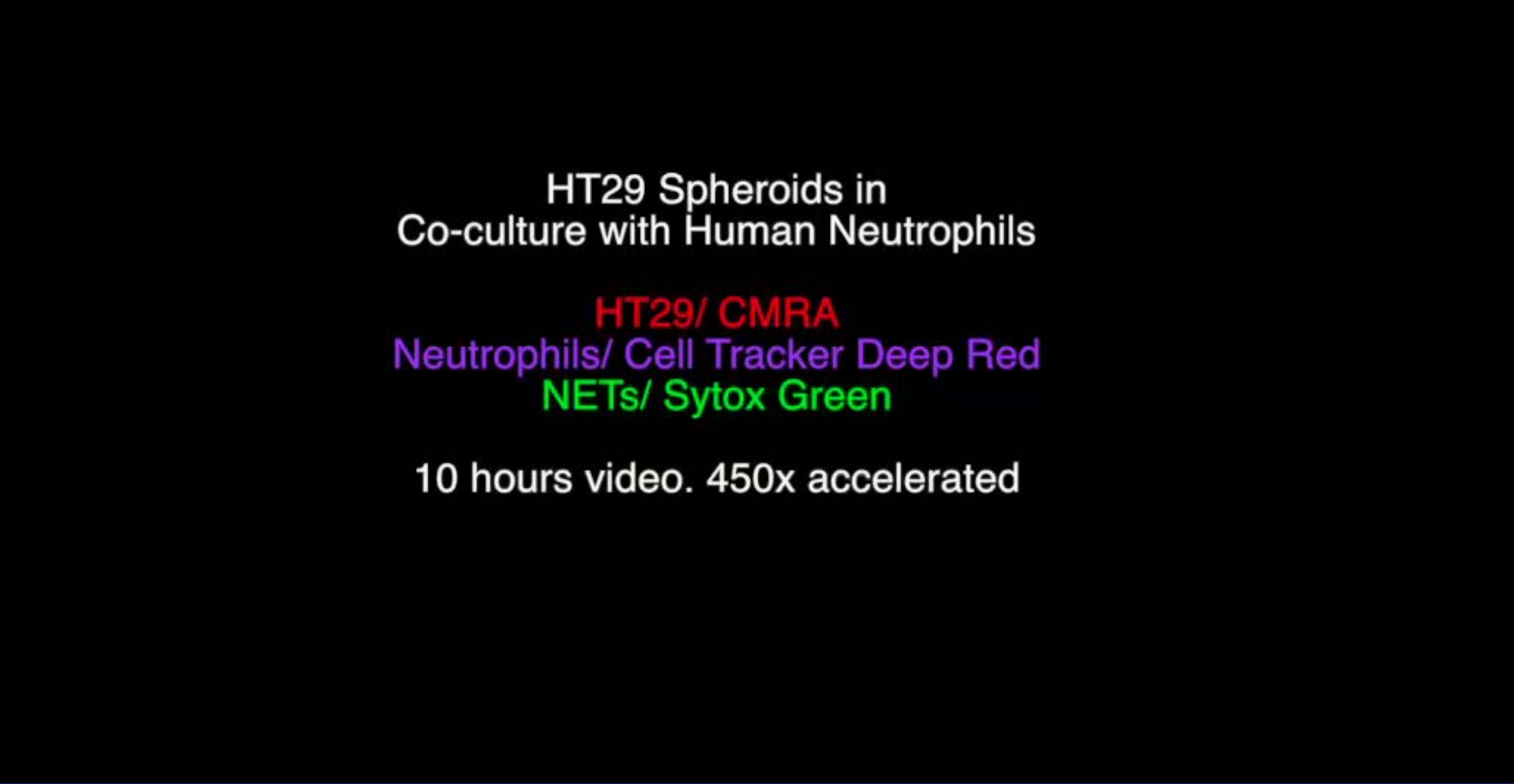
Fig.2 Tumor-derived Factors Induce NETosis in Neutrophils and GR-MDSCs by Activating CXCR1 and CXCR2 Receptors





Co-culture of 3D spheroids with Neutrophils induced NETosis

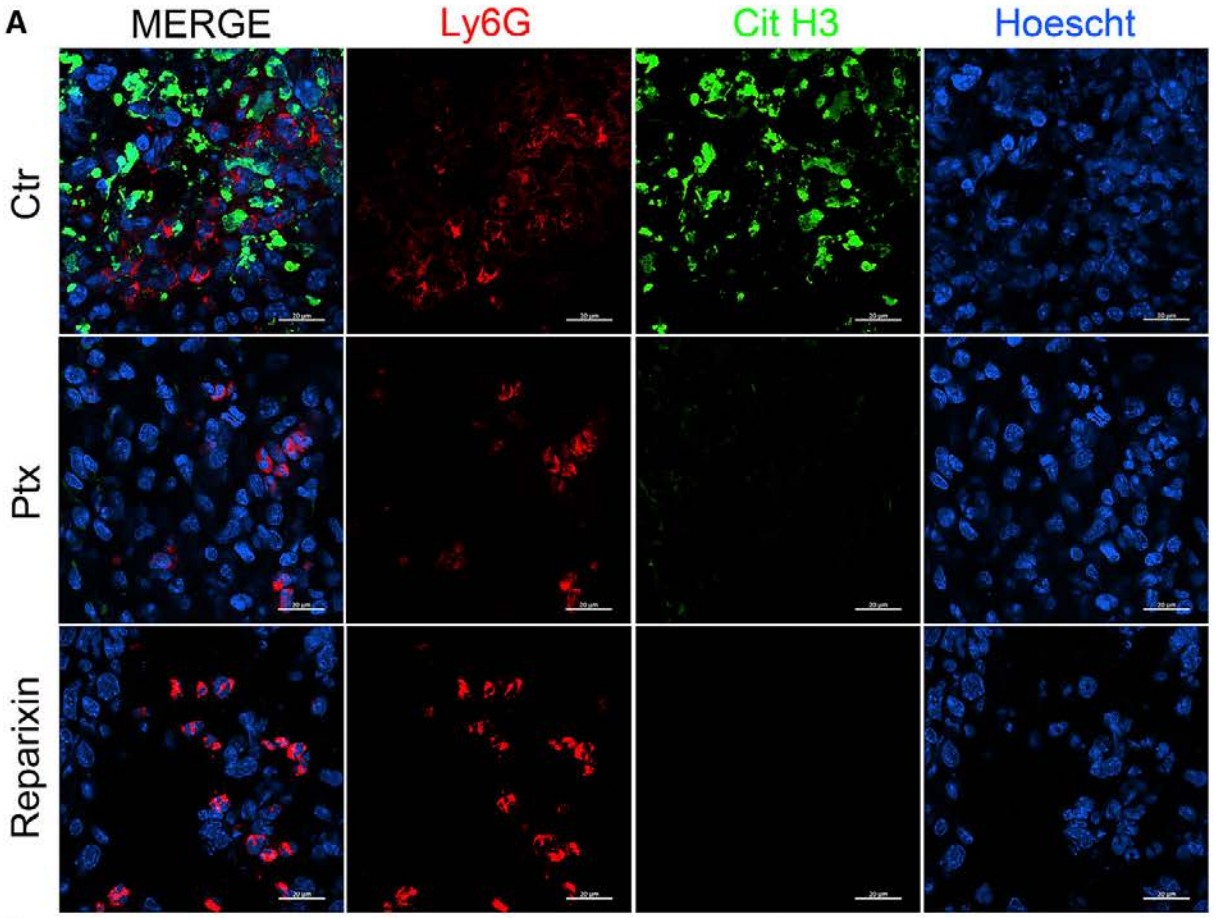
NETosis could be prevented by Reparixin treatment

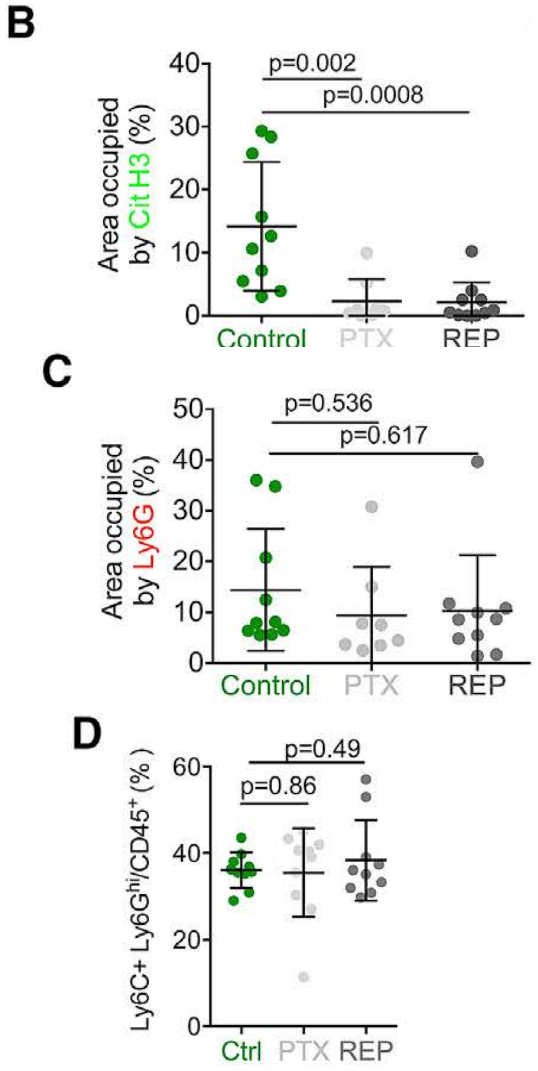

HT29 Spheroids in
Co-culture with Human Neutrophils
HT29/ CMRA
Neutrophils/ Cell Tracker Deep Red
NETs/ Sytox Green
10 hours video. 450x accelerated

To visualize the presence of NETs inside tumors

- 4T1 bearing mice
  - treated for 20h with either Ptx or Reparixin
- citH3 & Ly6G staining in tumor tissue samples

Fig. 3 Tumors Induce NETosis by activation of CXCR1 and CXCR2 Chemokine Receptors





a. Ptx and Reparixin
b. treatment sign. Reduced NETs presence within tumors

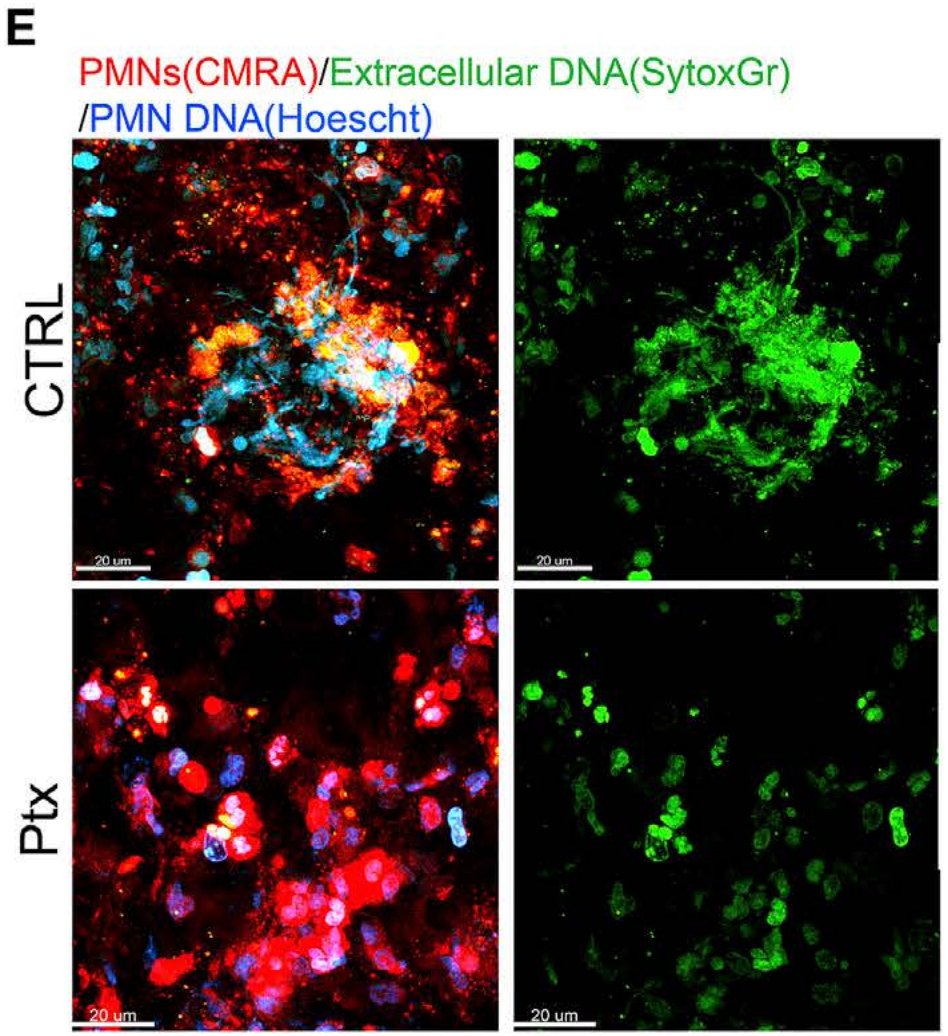
c. CXCR1 /CXCR2 inhibition did not alter GR-MDSC infiltrates

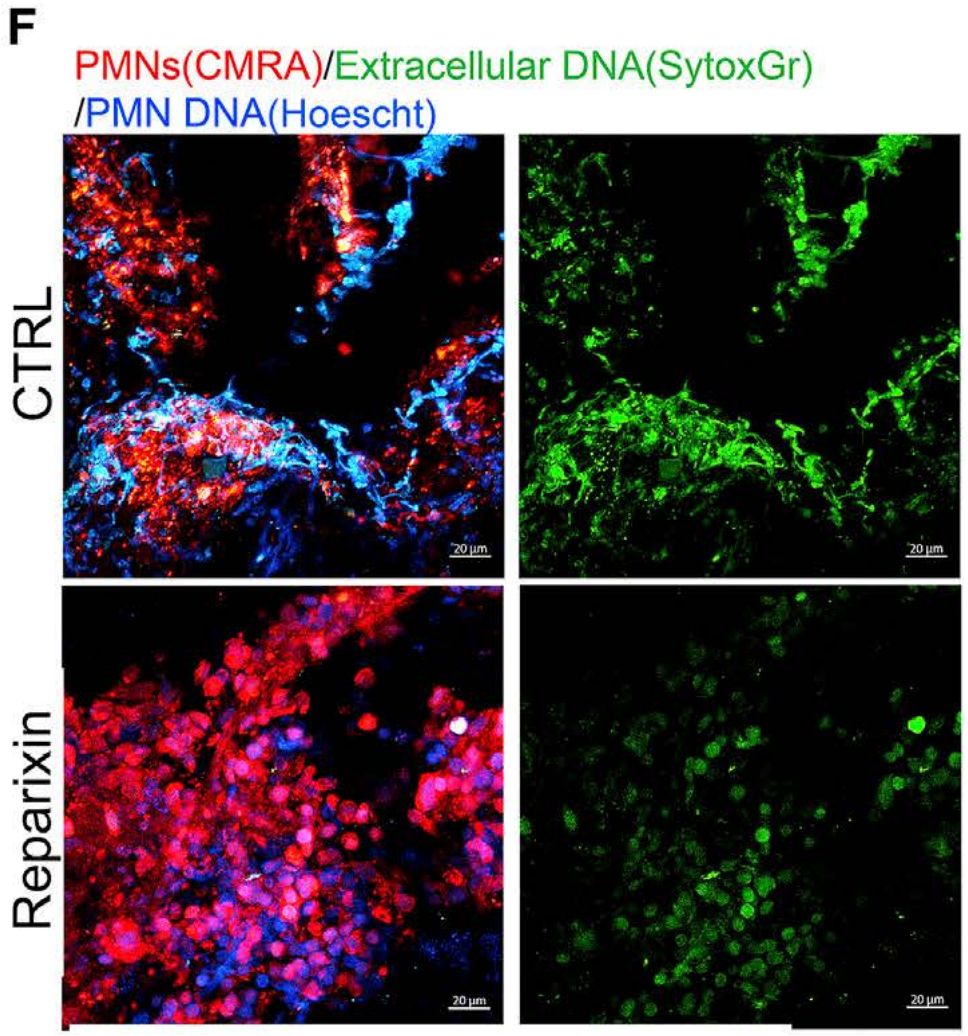
d. GR-MDSC presence was not altered by Ptx or Rep treatment

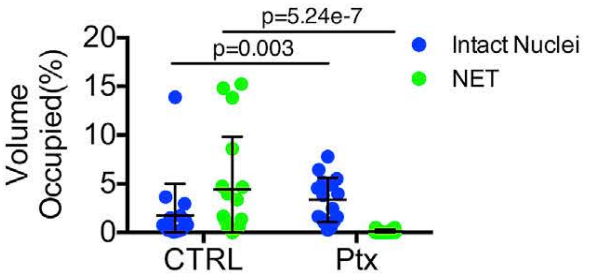
Can human tumors induce NETosis?

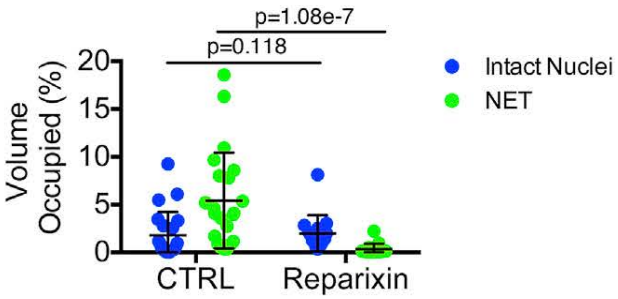
- Subcutaneous xenograft of HT29 tumor cells in Rag2$^{-/-}$ IL2rg$^{-/-}$ mice (lacking T-cells and NK-cells)
- Human pre-stained Neutrophils were injected intratumorally
  +/- Ptx
  +/- Reparixin
- 24h later → tumors were excised and analyzed
- 5min prior euthanasia: mice received SYTOX green systemically

Fig. 3 Tumors Induce NETosis by activation of CXCR1 and CXCR2 Chemokine Receptors







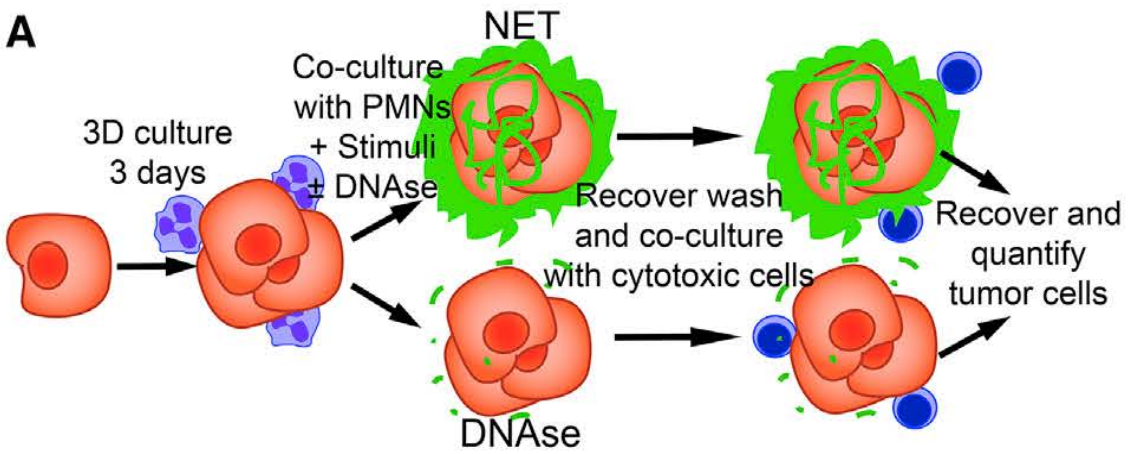


Is cytotoxicity impaired by NETs shielding cancer cells?

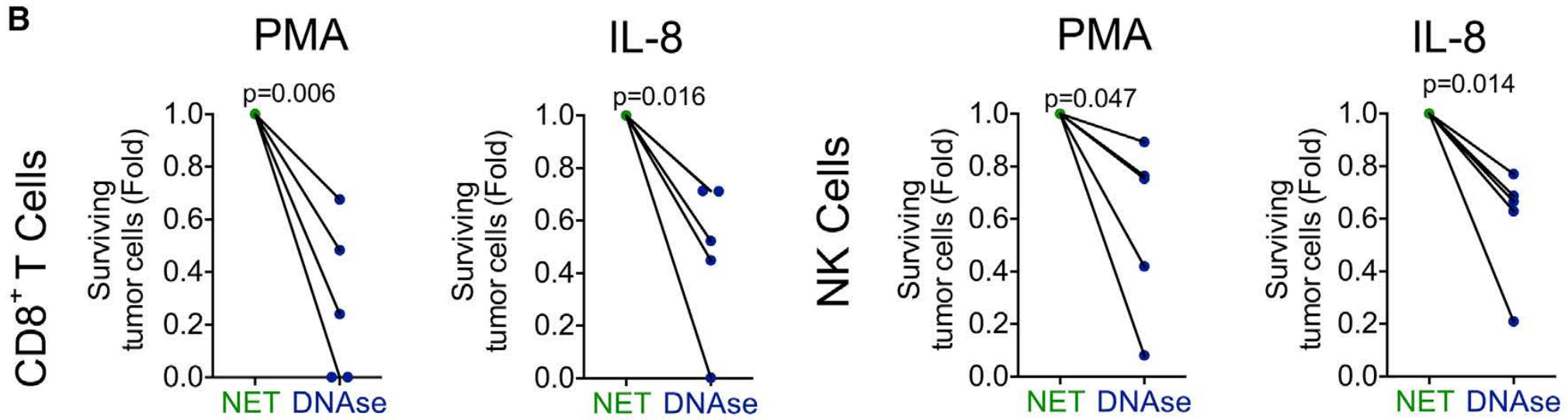
- 3D spheroids grown for 3 days
- Neutrophils added to spheroids
  +IL-8 / +PMA to induce NETosis
  or as control: +IL-8 / +PMA, + DNaseI

- Co-culture of effector cytotoxic lymphocytes with NET-covered tumor cells
  - IL-15 activated NK cells
  - CD3 plus CD28 activated $CD8^+$ T cells

Fig. 4 NETs Inhibit Immune Cell Cytotoxicity by Impeding Contact with Tumor Cells



Higher numbers of surviving tumor cells in presence of NETs
independent of NET-stimulus or effector lymphocytes



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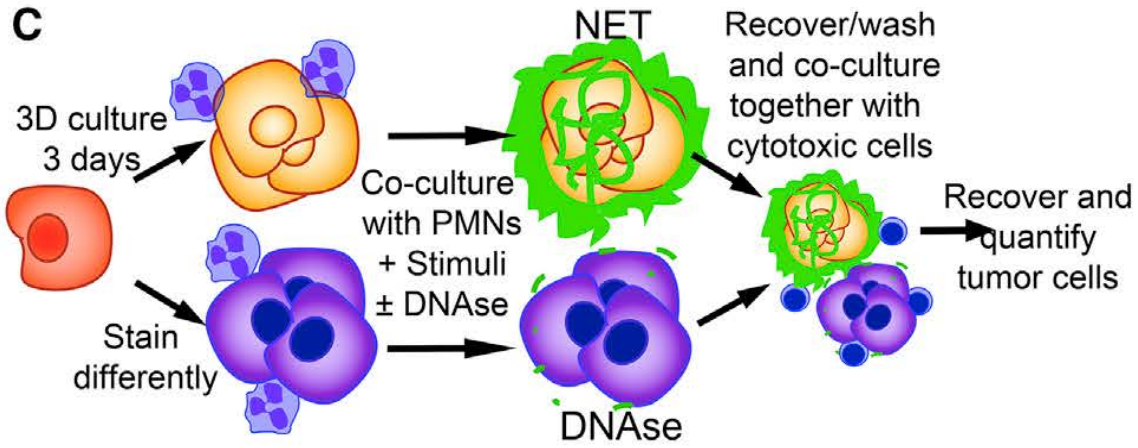
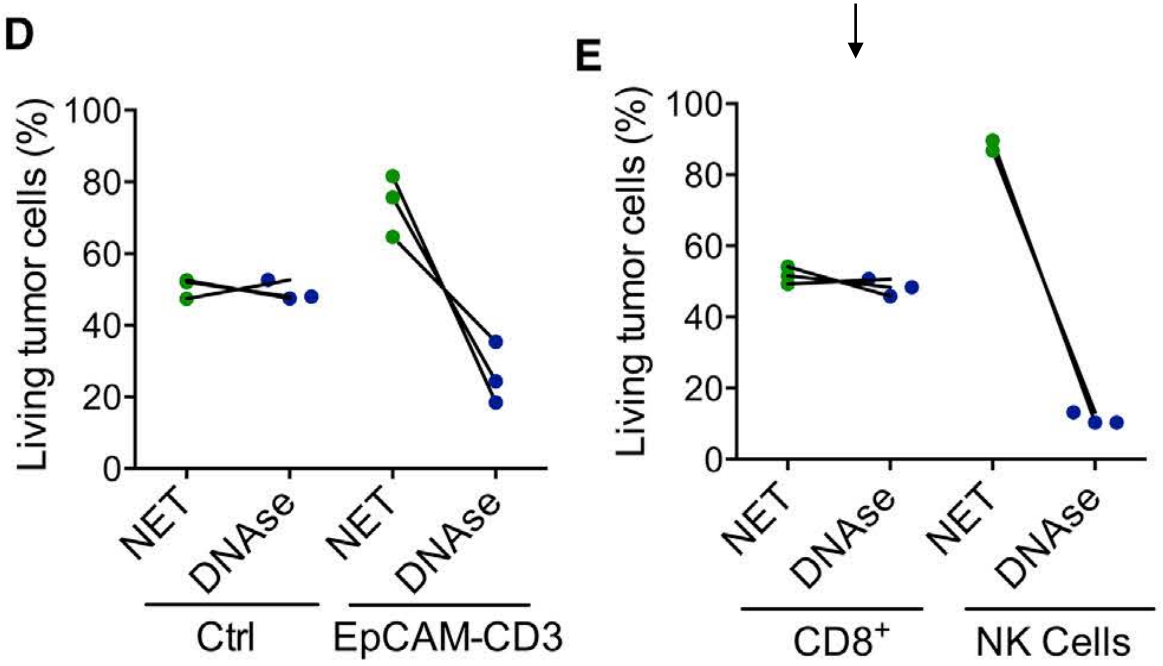


>80% of tumor cells survived in cytotoxic lmyphocyte co-cultures WHEN covered in NETs

No T cell bi-specific engager (EpCAM-CD3) = no cytotoxicity

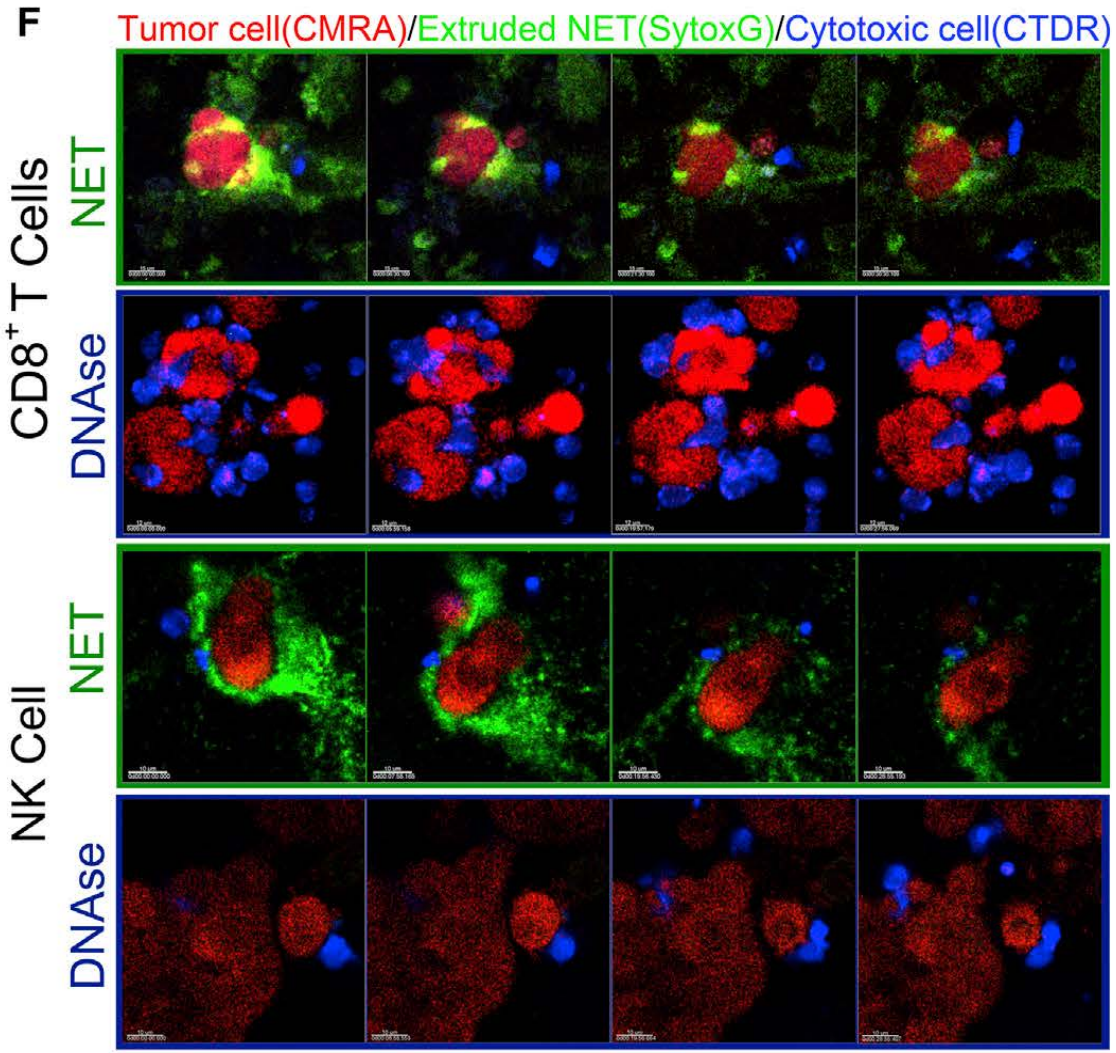


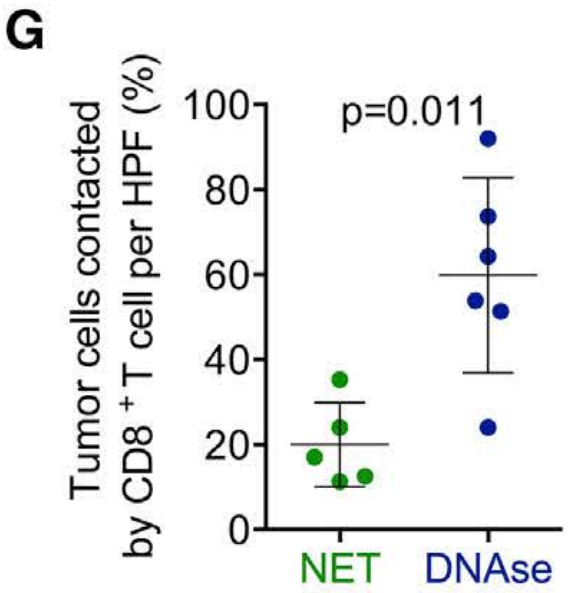
Do NETs shield tumor cells from contact with cytotoxic immune cells?

- Time-lapse confocal microscopy (same co-cultures as before)

- NETs generated over chemotaxis transwells
  to see whether they directly impari $CD8^+$ T cell migration

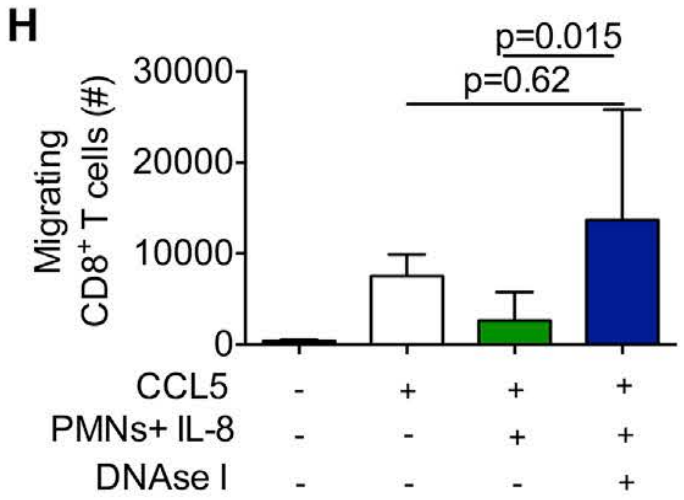
Fig. 4 NETs Inhibit Immune Cell Cytotoxicity by Impeding Contact with Tumor Cells

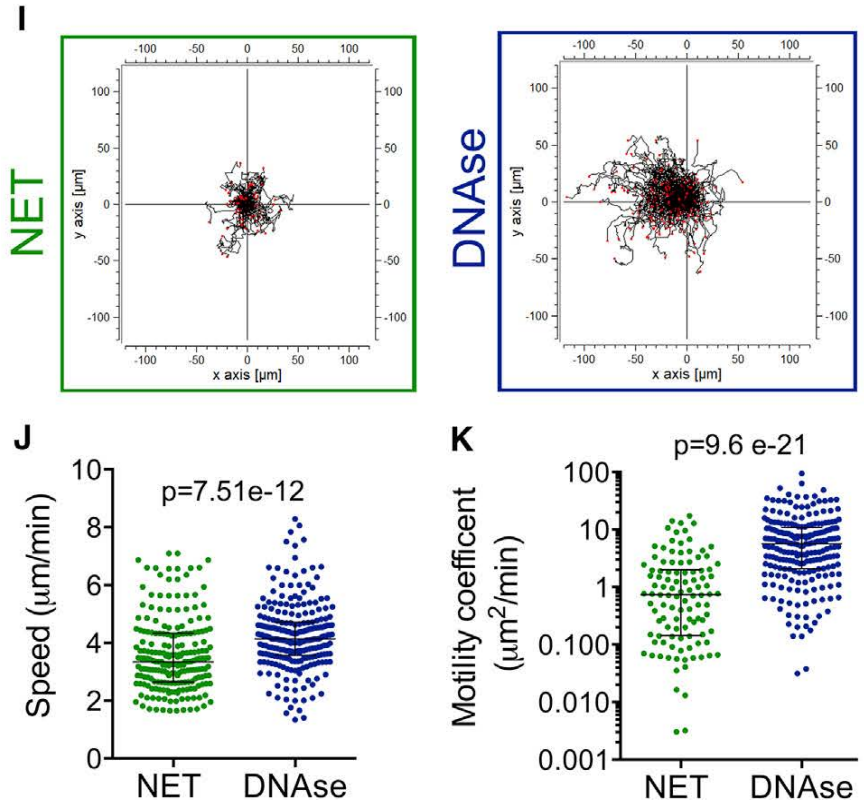




less lymphocyte-tumor cell contacts in presence of NETs

Fig. 4 NETs Inhibit Immune Cell Cytotoxicity by Impeding Contact with Tumor Cells





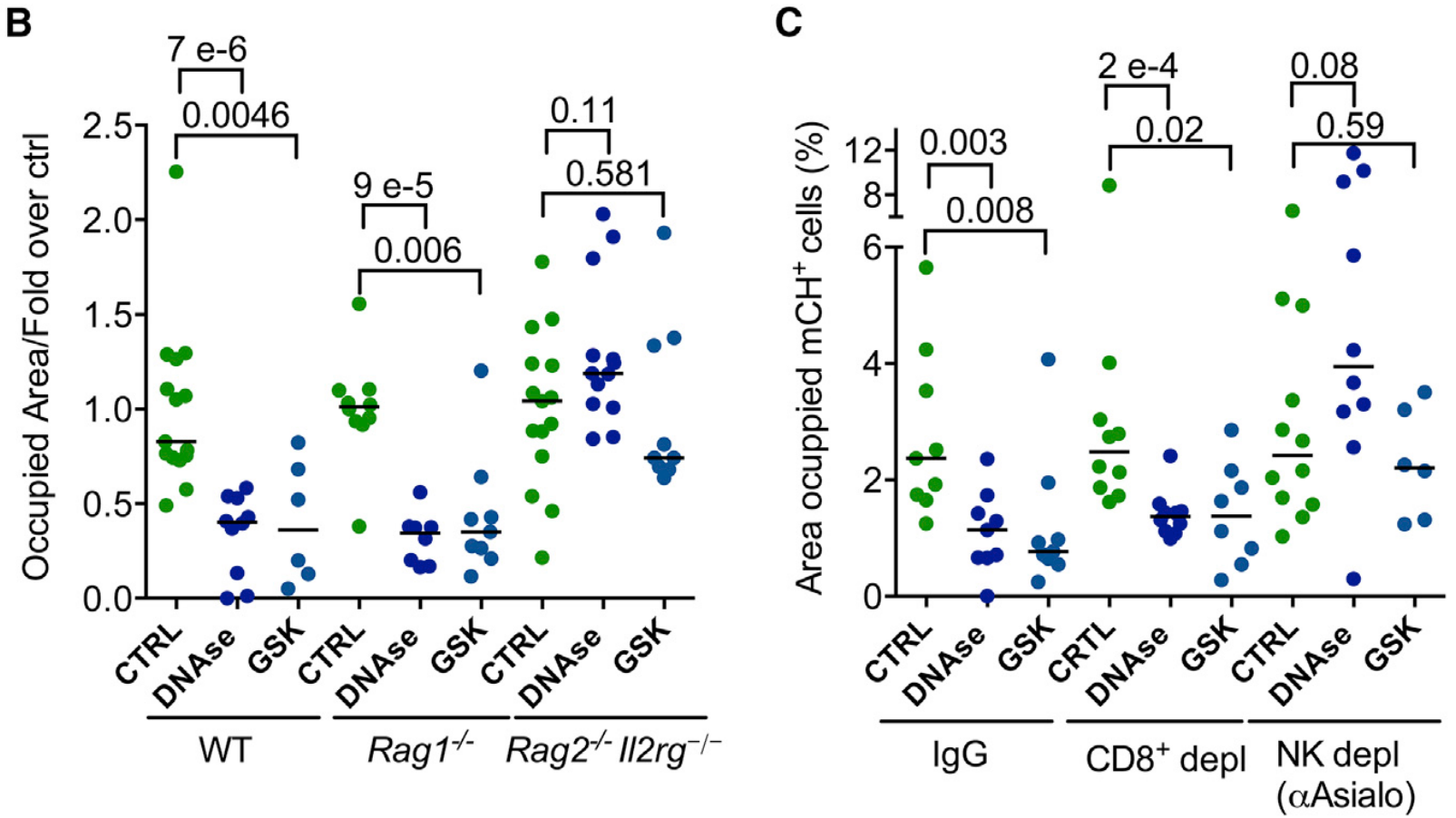
Less CD8+ T cell migration over chemotaxis transwells towards CCL5 in presence of NETs

Restoration of chemotaxis by DNaseI treatment

Do NETs influence immune-cell control of tumor metastasis?

- Lung metastasis mouse model in mice bearing 4T1 bilateral tumors
  - WT BALB/C
  - Rag1 $^{-/-}$ (lack T cells)
  - Rag2 $^{-/-}$ IL2rg $^{-/-}$ (lack T cells and NK cells)
- Intravenous injection of 4T1 mCherry tagged tumor cells

Fig. 5 NETs Limit Immune Response and Checkpoint-Based Immunotherapy against 4T1 Tumors

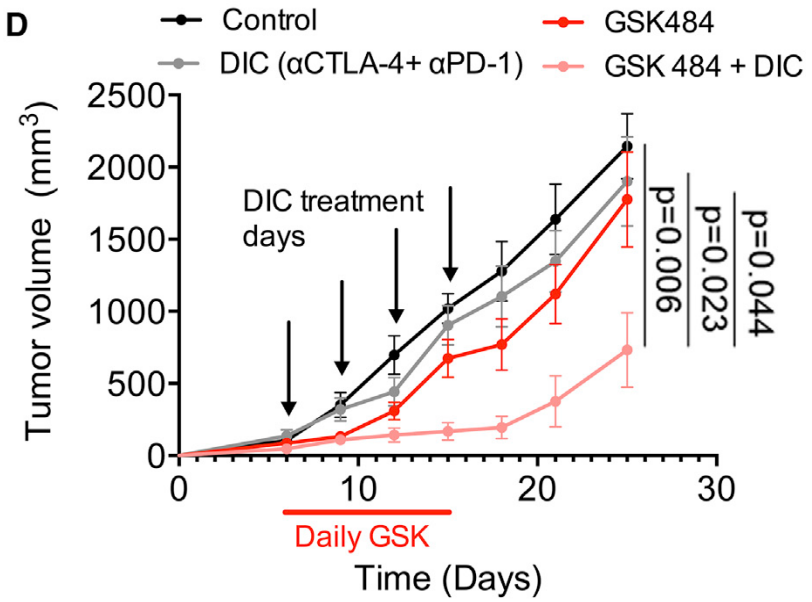


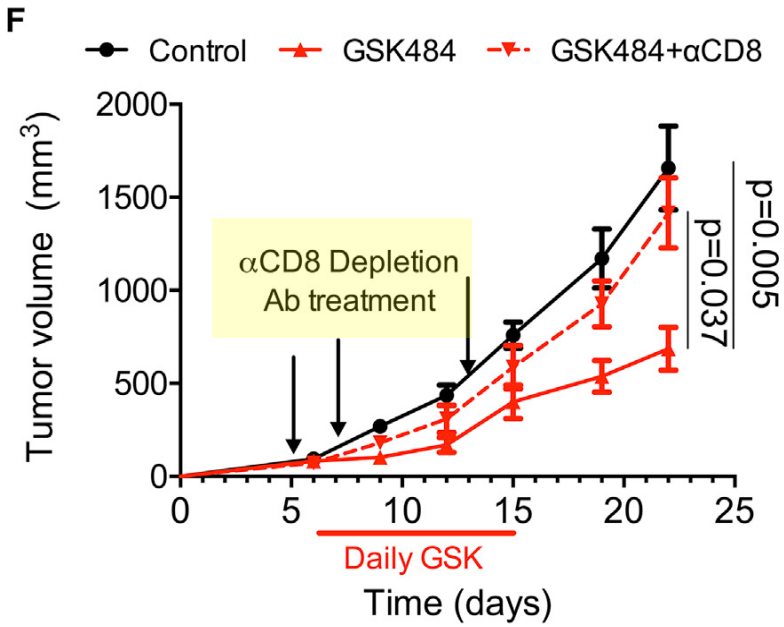
DNaseI treatment and PAD4 inhibition could sign. reduce the number of lung mCherry^{+} 4T1 micrometastases 24h post IV injection
in **WT** and **Rag1-/-** but **not in Rag2$^{-/-}$ IL2rg$^{-/-}$** mice

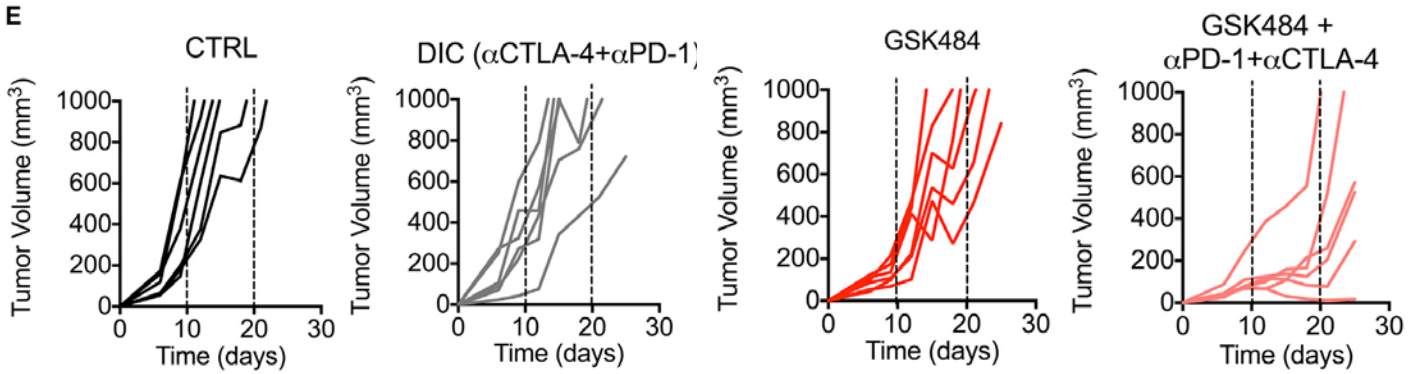
Selective NK cell depletion showed similar results

→ **Indication that NK cells are capable of controlling early stages of metastases**

Fig. 5 NETs Limit Immune Response and Checkpoint-Based Immunotherapy against 4T1 Tumors







IP treatment with PAD4 inhibitor minimally decreased tumor progression

Combination of PAD4 inhibitor and anti-PD-1 plus anti-CTLA-4 checkpoint inhibitors showed sign. reduction in tumor progression

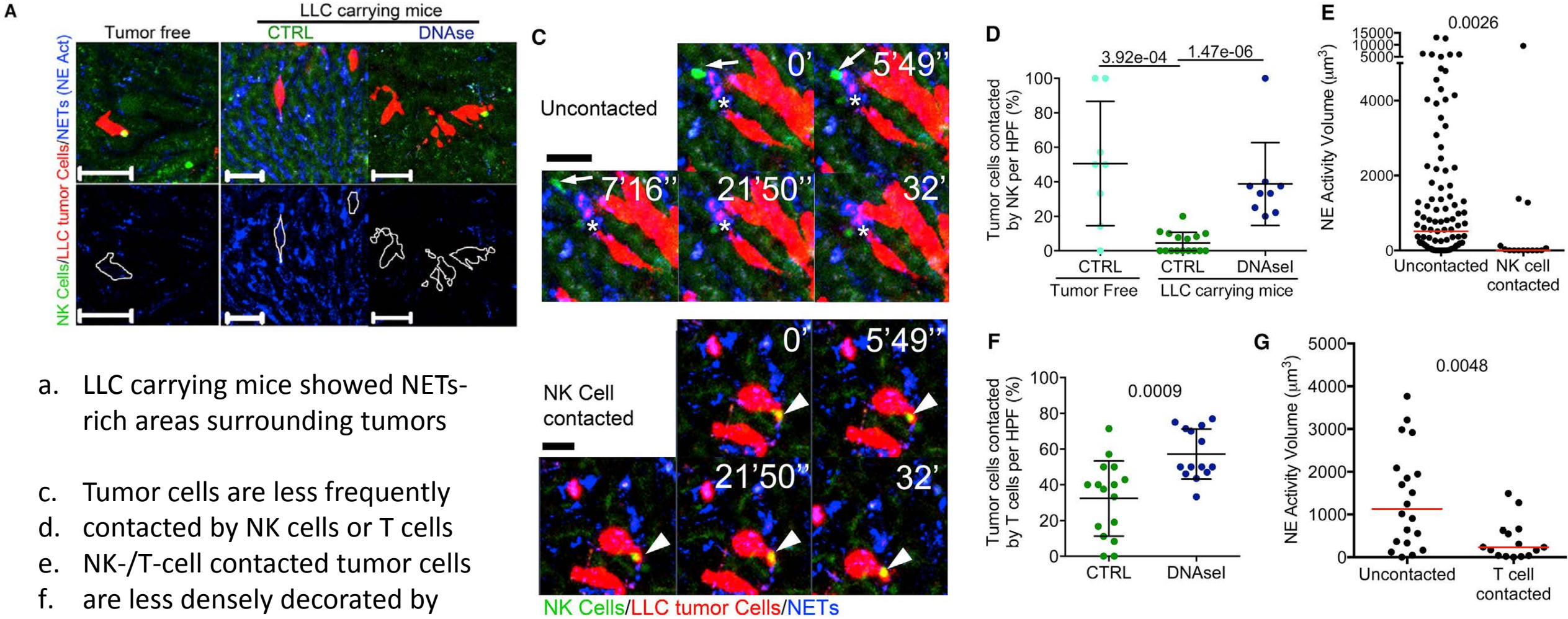
PAD4-immune checkpoint tumor suppression is $CD8^+$ T cell dependent

Intravital microscopy of NETs imparing NK and T cell –tumor contact

- IVM of liver and subcutaneous tumors located in ear dermis
- Mice with subcutaneous Lewis-Lung-Carcinoma (LLC) tumors received fluorescent labelled LLCs intrasplenically

- Similar experiment but in recipient mice with GFP labelled NK cells or RFP labelled T cells

Fig. 6 NETs Impair Cytotoxic Cell Contact with Tumor Cells in the Metastatic Intravascular Niche



a. LLC carrying mice showed NETs-rich areas surrounding tumors

c. Tumor cells are less frequently
d. contacted by NK cells or T cells
e. NK-/T-cell contacted tumor cells
f. are less densely decorated by
g. NETs

IVM simultaneous comparison of CTLs in NET-rich and NET-lacking areas

- 4T1 mCherry tumors implanted into ears of mice carrying 4T1 tumors in the flank
- Due to uneven SYTOX (=NETs) distribution → simultaneous comparison of CTL behavious possible
- Injection of B16OVA-H2BmCherry tumors in ears of mice adoptively transferred fluorescently labelled OT-I $CD8^+$ T cells (recognizing OVA)
- Direct injection with human pre-stained NETs

Fig. 7 NETs Impair Cytotoxic Cell Contact with Tumor Cells in Subcutaneous Tumors

Uneven NETs distribution; not necessarily coating tumor cells

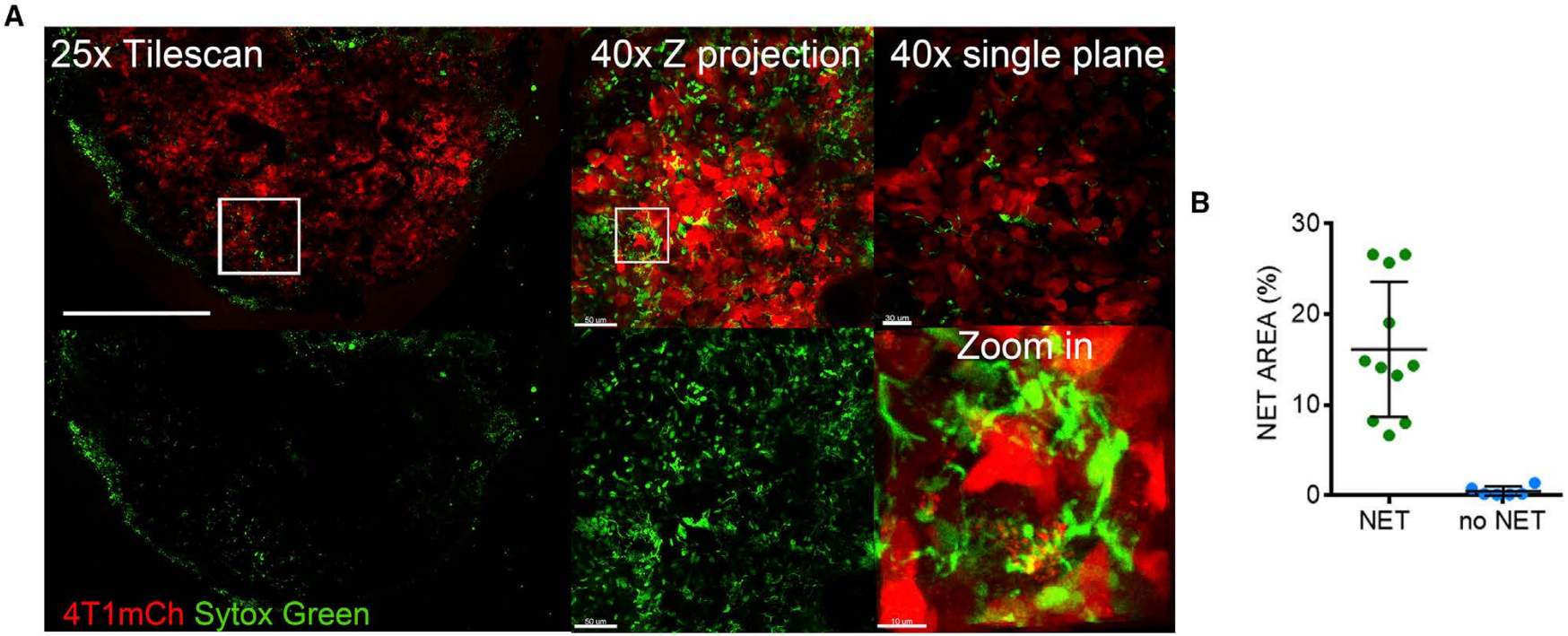
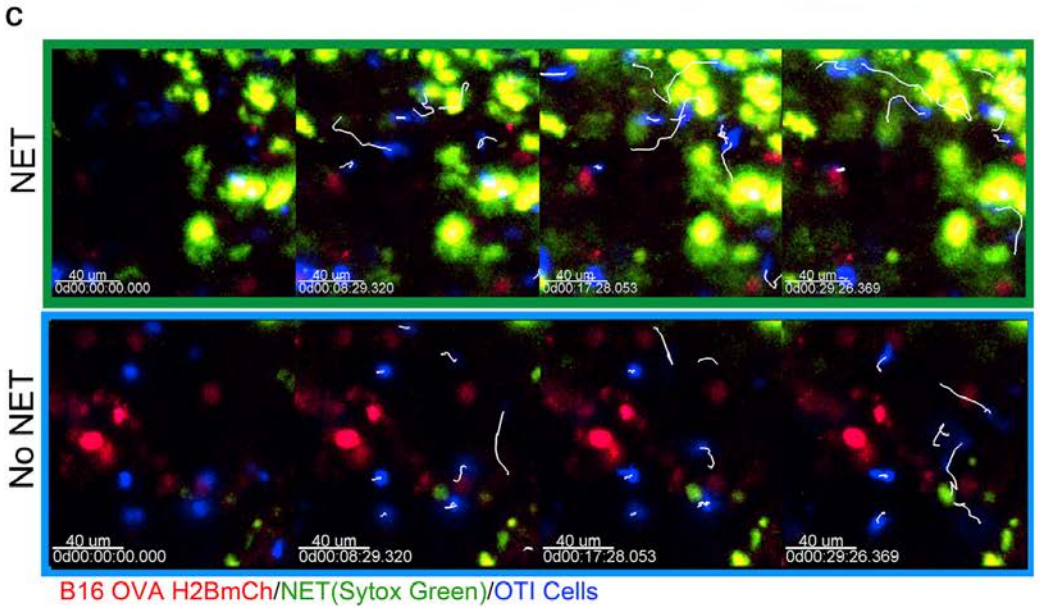
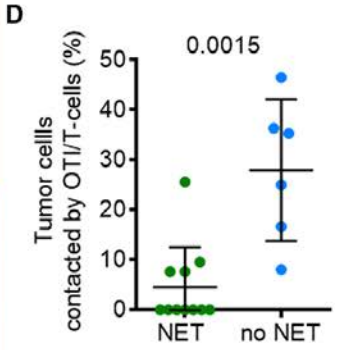


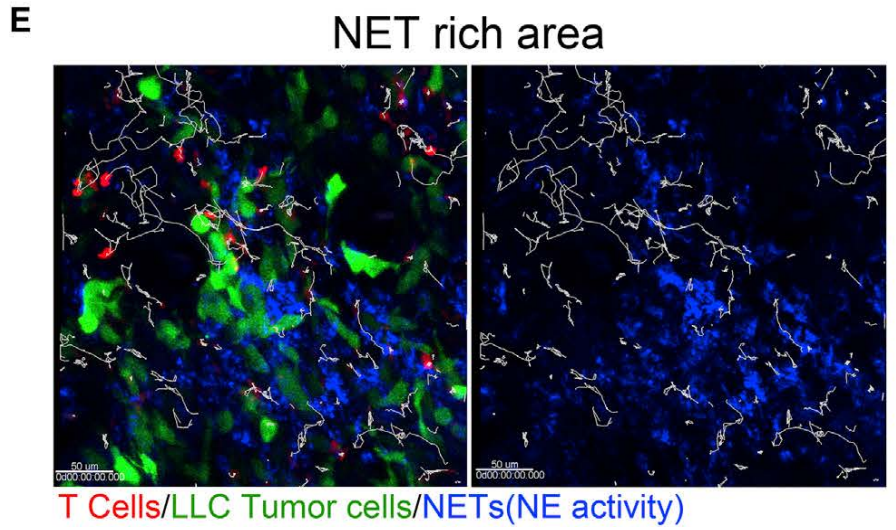
Fig. 7 NETs Impair Cytotoxic Cell Contact with Tumor Cells in Subcutaneous Tumors

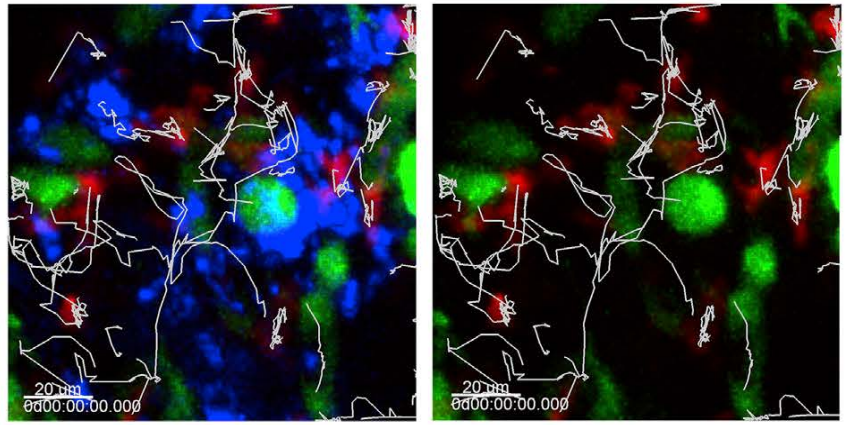
NETs surrounding tumor cells prevented OTI cells from contacting tumor cells

NET-rich areas showed imparid T cell-tumor cell contact







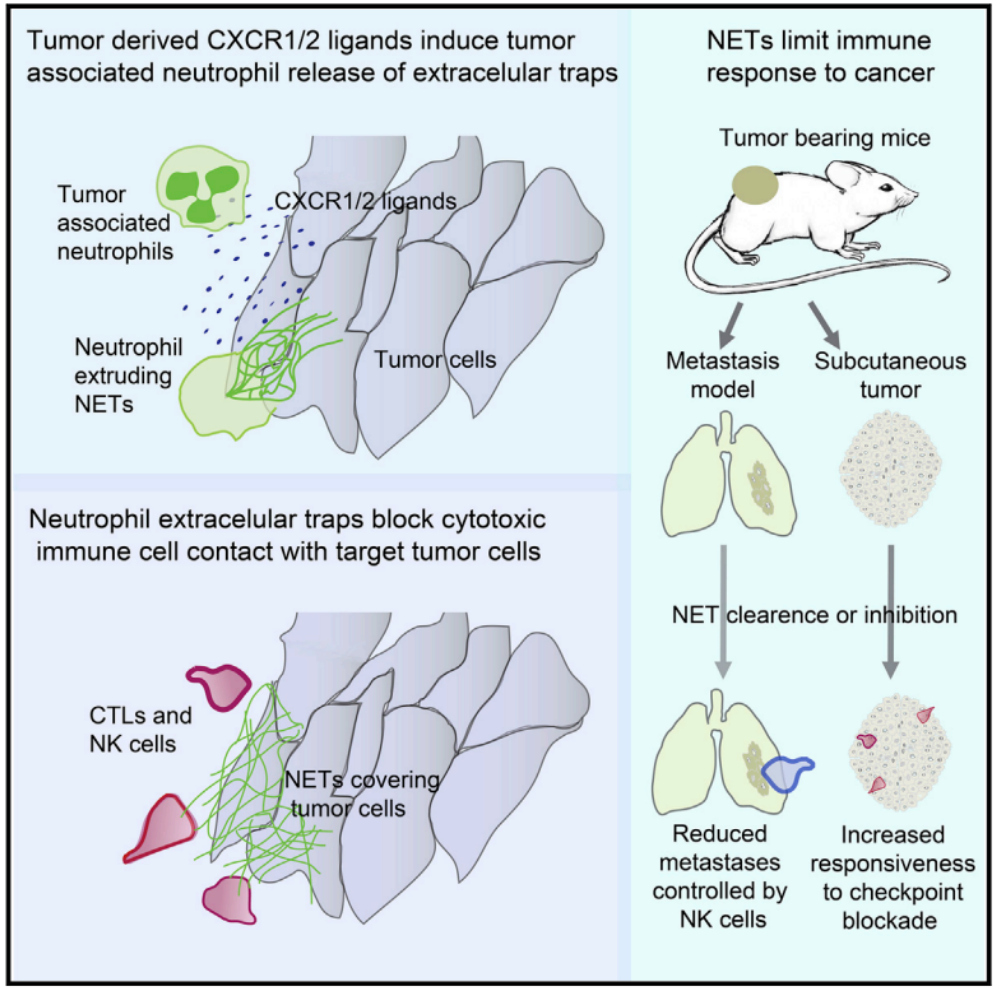


LLC GFP tumor cells (green) implanted in the ear of hCD2RFP mice (T-cells, Red) in the presence of NE fluorescent substrate to visualize NETs(blue)

Discussion

- Tumor-derived CXCR1 and CXCR2 ligands trigger NETosis in human and mouse Neutrophils and GR-MDSCs

- NETs protect tumor cells from cytotoxic T lymphocytes and NK cytotoxicity



Discussion

- Inhibition of NETosis, via PAD4 inhibition, sensitizes tumors to immune checkpoint therapy (PD-1+CTLA-4)

- NETs impair contact of immune cells with tumor cells in mice