Supplementary Information

Behaviorome profiling of anti-tumor and pro-tumor human neutrophil subtypes in a microphysiological system

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Supplementary Figures

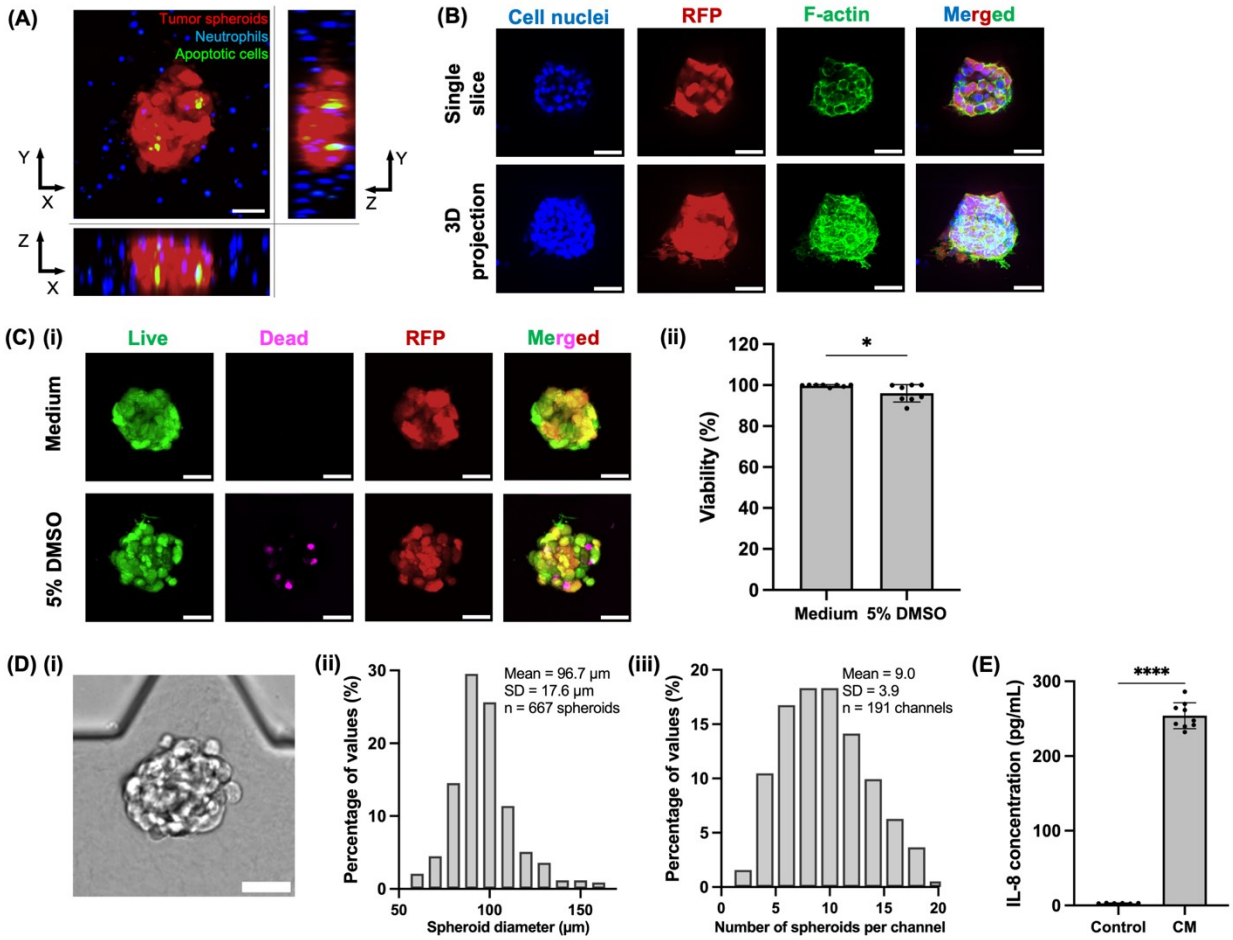


Figure S1. Validation and characterization of the phenotypes of RFP-PANC-1 tumor spheroids. (A) Representative 10X confocal images showing the top (X-Y), side (Y-Z), and front (X-Z) views of a tumor spheroid (red, RFP) surrounded and infiltrated by neutrophils (blue, BMQC Violet) in the NTI-chip. Apoptotic cells were stained with Caspase-3/7 Green. Images are maximum intensity projections of z-stacks with a 2 µm step size. Scale bar, 50 µm. **(B)** Representative 20X confocal images showing a tumor spheroid (red, RFP) stained for cell nuclei (blue, Hoechst) and F-actin (green, phalloidin) in the NTI-chip. Images were single slices (top panel) or maximum intensity projections (bottom panel) of z-stacks with a 2 µm step size. Scale bar, 50 µm. **(C) (i)** Representative 10X confocal images showing tumor spheroids (red, RFP) stained for live cells (green, Calcein AM) and dead cells (magenta, SYTOX Deep Red) using the LIVE/DEAD™ Viability/Cytotoxicity Assay Kit after 24 h of culture in medium alone or 5% DMSO (positive control) in the NTI-chip. Images were maximum intensity projections of z-stacks with a 2 µm step size. Scale bar, 50 µm. **(ii)** Bar plot showing the viability of tumor spheroids, quantified as the percentage of live volume per spheroid, in the culture medium and 5% DMSO conditions. n= 8 spheroids per condition from one experiment. *: $p<0.05$, unpaired t test. **(D) (i)** A representative 10X brightfield image of a tumor spheroid embedded in collagen gel matrix in the NTI-chip. Scale bar, 50 µm. **(ii)** The frequency distribution of diameters of tumor spheroids loaded into the two side channels of the NTI-chip. The mean diameter is 96.7 ± 17.6 µm. n= 667 spheroids. **(iii)** The frequency distribution of the number of tumor spheroids loaded into each side channel of the NTI-chip. The mean number is 9.0 ± 3.9. n = 191 side channels. **(E)** Concentrations of neutrophil chemoattractant IL-8 in the conditioned medium (CM) of tumor spheroids cultured on ULA plates for 2 days and culture medium control were measured by ELISA. CM samples were collected from three independent experiments. ****: $p<0.0001$, unpaired t test.

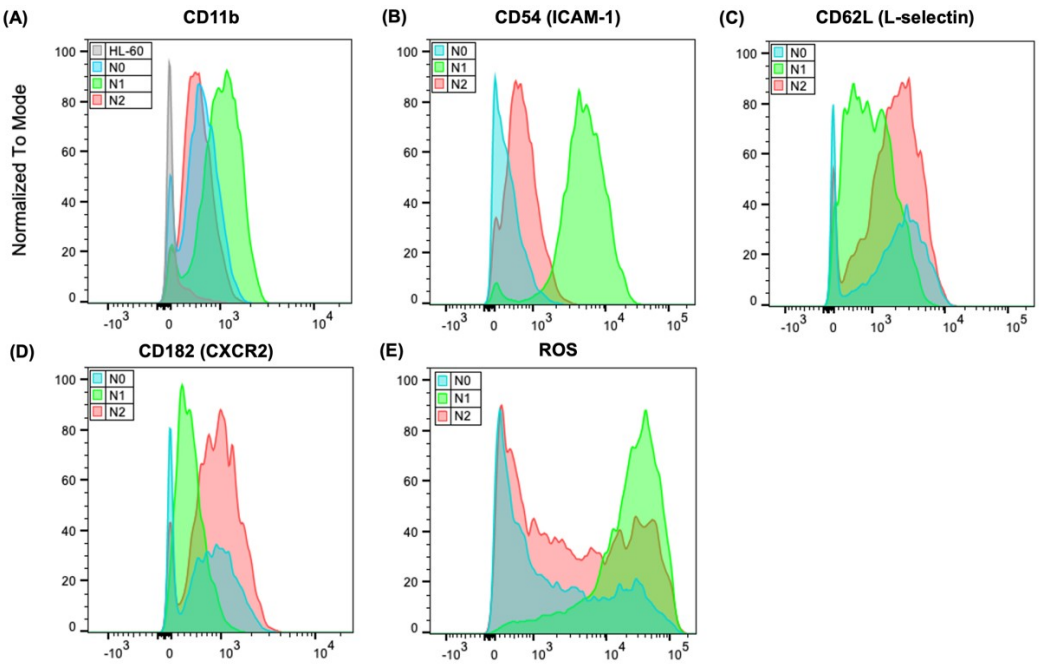


Figure S2. Representative histograms of surface marker expressions and ROS production by N0, N1-like, and N2-like neutrophils. Expressions of CD11b **(A)**, CD54 (ICAM-1) **(B)**, CD62L (L-selectin) **(C)**, and CD182 (CXCR2) **(D)** and production of reactive oxygen species (ROS) **(E)** by N0, N1-like, and N2-like neutrophils were examined using flow cytometry. CD11b expression by undifferentiated HL-60 cells was also examined as a negative control. Gray histogram = HL-60 cells; Blue histograms = N0 neutrophils; green histograms = N1-like neutrophils; red histograms = N2-like neutrophils. CD11b is a general neutrophil marker. Typical N1 surface markers are $CD54^{high}$, $CD62L^{low}$, and $CD182^{low}$, while typical N2 surface markers are $CD54^{low}$, $CD62L^{high}$, and $CD182^{high}$. ROS is a pro-inflammatory marker and a cytotoxic mediator. Histograms are from one representative flow cytometry experiment for each marker.

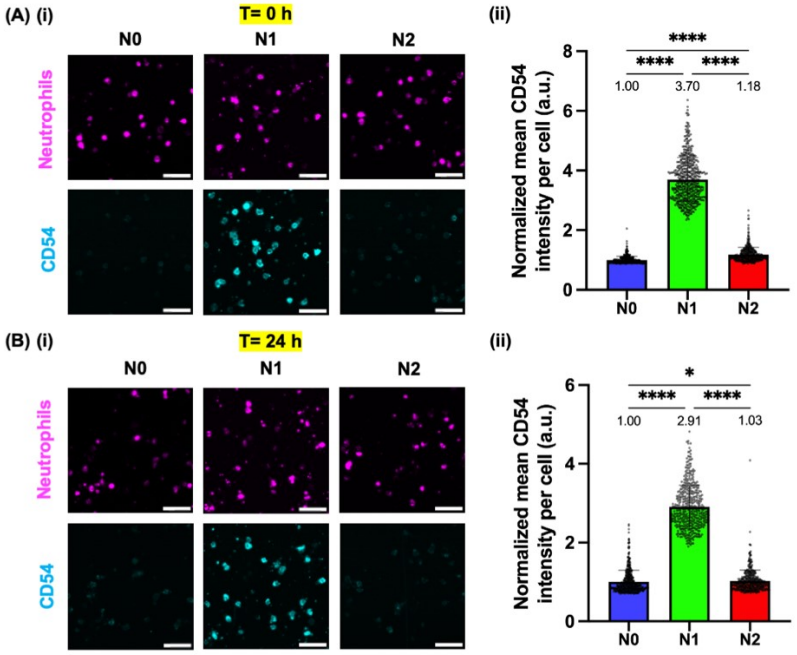


Figure S3. N1-like and N2-like neutrophils maintain their polarization states for at least 24 h of incubation in the NTI-chip. N1-like neutrophils maintained a significantly higher expression level of typical N1 marker CD54 than N2-like neutrophils throughout 24 h. **(A) (i)** Representative 10X confocal images of N0, N1-like, and N2-like neutrophils (DiD, magenta) immunostained for CD54 (ICAM-1) (blue) at t=0 h after being embedded in collagen hydrogel and loaded into the NTI-chip. Images are maximum intensity projections of z-stacks with a 2 µm step size. Scale bar, 50 µm. **(ii)** Bar plot showing the mean intensity of CD54 per neutrophil in specified conditions at t=0 h. Raw values were normalized by the mean of the N0 condition. n = 430-556 neutrophils per condition. Bars show mean ± SD with the mean values written above the points. **(B) (i)** Representative 10X confocal images of N0, N1-like, and N2-like neutrophils (DiD, magenta) immunostained for CD54 (ICAM-1) (blue) at t=24 h after being embedded in collagen hydrogel and loaded into the NTI-chip. **(ii)** Bar plot showing the mean intensity of CD54 per neutrophil in specified conditions at t=24 h. n = 443-525 neutrophils per condition. The experiment was repeated twice. *: $p<0.05$, ****: $p<0.0001$, Kruskal-Wallis test.

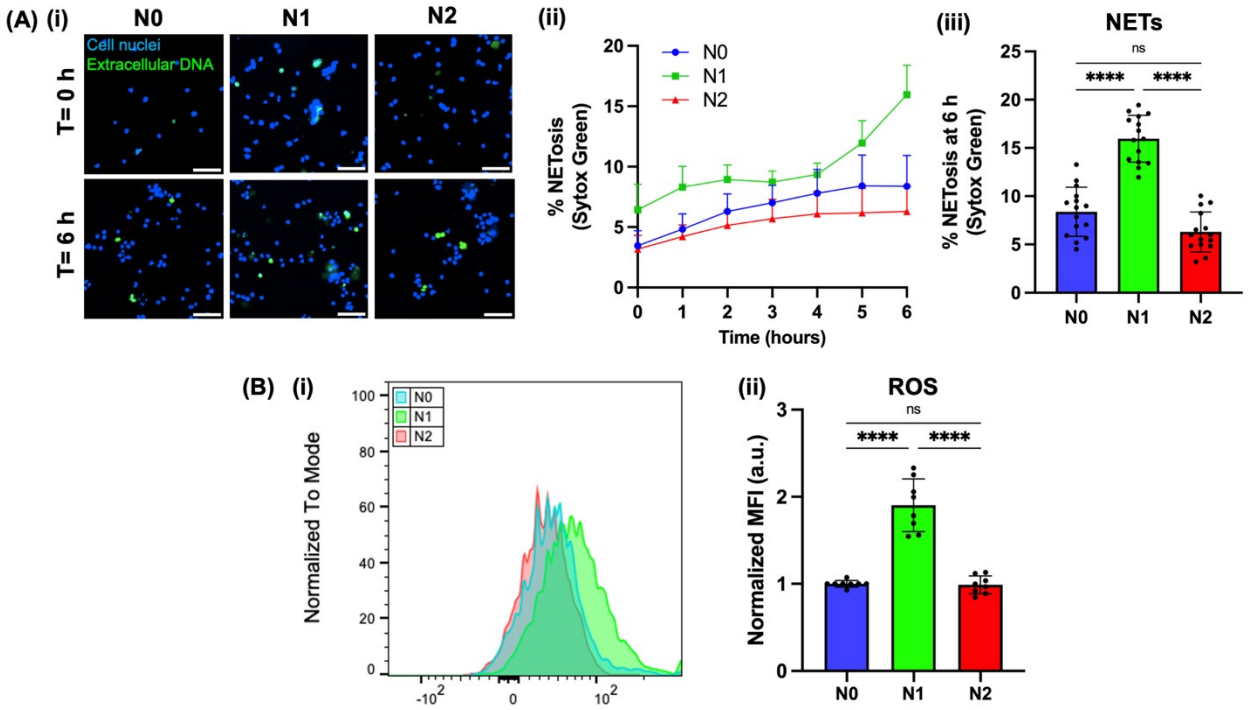


Figure S4. N1-like neutrophils show higher baseline levels of NET release and ROS production than N2-like neutrophils without PMA stimulation. (A) (i) Representative 20X images showing the release of neutrophil extracellular traps (NETs) (Sytox Green, extracellular DNA) by N0, N1-like, and N2-like neutrophils (Hoechst blue, nuclei) treated with DMSO vehicle control without PMA on a 96-well plate at t=0 h and 6 h. Scale bar, 50 µm. **(ii)** Temporal dynamics of NET release over 6 h. **(iii)** A bar plot showing the percentage of NET-releasing neutrophils at t= 6 h. Bars show mean ± SD of n= 15 random ROIs per condition. **(B)** Reactive oxygen species (ROS) production by N0, N1-like, and N2-like neutrophils treated with DMSO vehicle control was assessed by flow cytometry. **(i)** Histograms of one representative experiment. Blue histogram = N0 neutrophils; green histogram = N1-like neutrophils; red histogram = N2-like neutrophils. **(ii)** Bar plot showing the normalized median fluorescence intensity (MFI) of intracellular ROS in N0, N1-like, and N2-like neutrophils. Raw values were normalized by the mean of the N0 condition. At least three independent experiments were performed. ns: ≥0.05, ****: p<0.0001, ANOVA with Tukey multiple comparisons test.

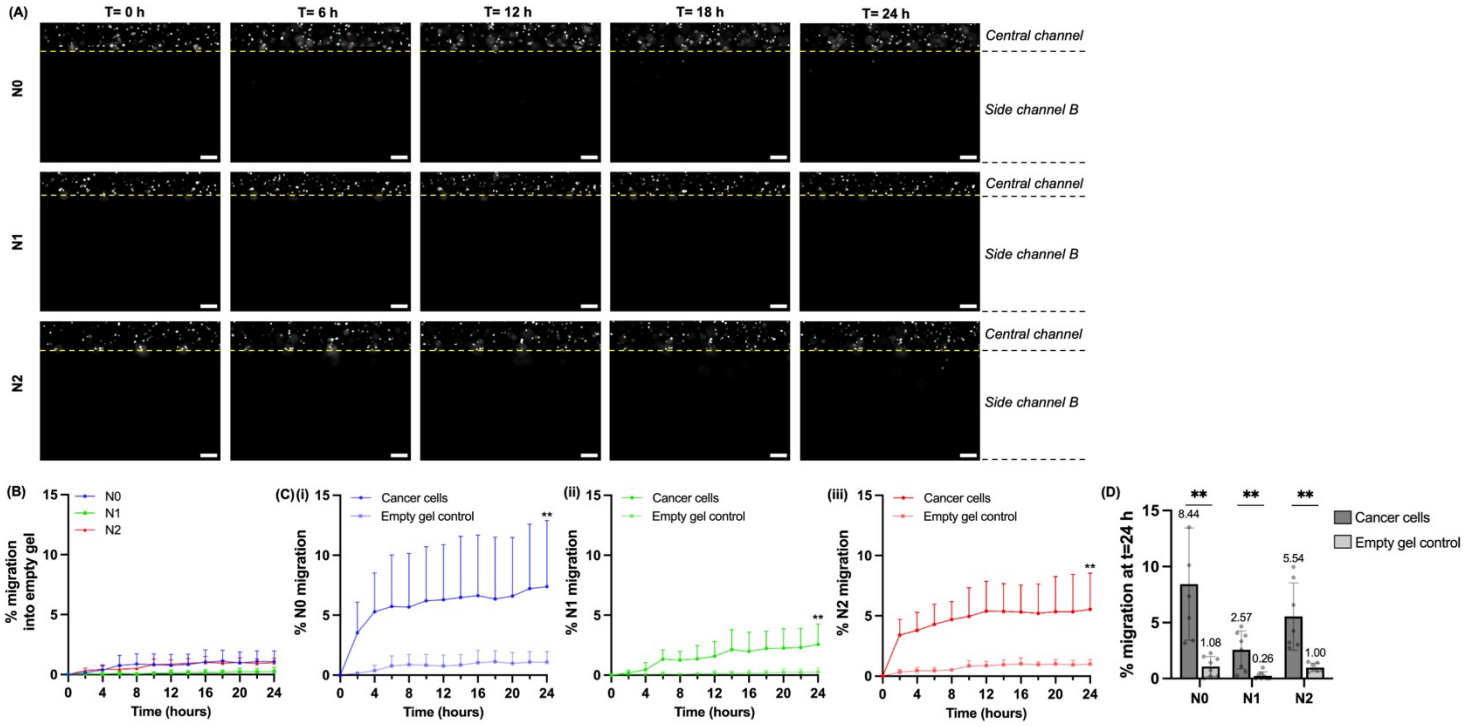


Figure S5. In scenario 1, N0, N1-like, and N2-like neutrophils show limited migration into the empty hydrogel as negative control. (A) Representative 10X images showing N0, N1-like, or N2-like neutrophils (gray) migrating from the central channel into side channel B housing empty collagen hydrogel at t=0 h, 6 h, 12 h, 18 h, and 24 h. The yellow dashed line marks the boundary bewteen the central channel and side channel B. Scale bar, 100 µm. **(B)** Line graph showing the percentage of neutrophil migration into empty gel, defined as the number of neutrophils in side channel B at a given time point divided by the initial number of neutrophils in the central channel at t=0 h, every 2 h over 24 h in specified conditions. Bars show mean + SD. **(C)** Line graphs showing the percentage of migration of N0 **(i)**, N1-like **(ii)**, and N2-like **(iii)** neutrophils into side channel A (cancer cells) versus into side channel B (empty gel control) every 2 h over 24 h. Bars show mean + SD. **(D)** Bar plot showing the percentage of neutrophil migration at t=24 h. Bars show mean ± SD with mean values written above the points. Each data point represents an NTI-chip and n= 8 chips per condition. At least four independent experiments were performed. **: $p<0.01$, unpaired t test.

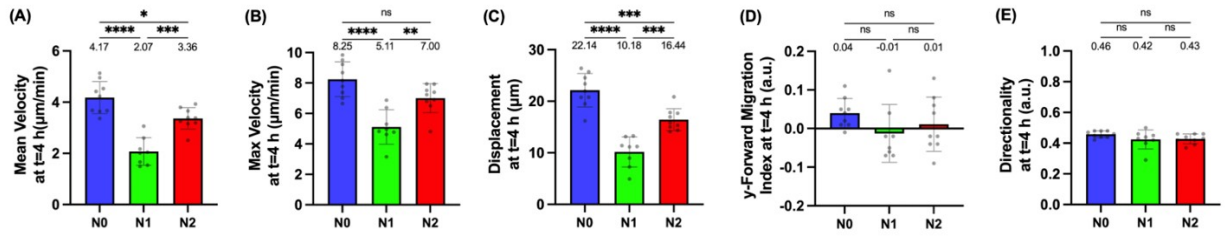
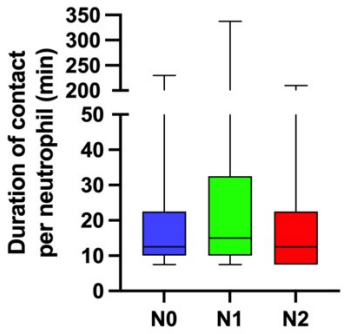


Figure S6. In scenario 1, N2-like neutrophils show higher motility than N1-like neutrophils after migration toward PANC-1 cancer cells at t=4 h. Bar plots showing the mean velocity **(A)**, maximum velocity **(B)**, displacement **(C)**, y-FMI **(D)**, and directionality **(E)** of N0, N1-like, and N2-like motile neutrophils at t= 4 h. Each data point represents the average value of all tracked neutrophils per NTI-chip and n= 9 chips per condition. Bars show mean ± SD with mean values written above the points. At least four independent experiments were performed. ns: $p>0.05$, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$, ordinary one-way ANOVA.

(A)



(B)

Neutrophil subtype	25% percentile	50% percentile	75% percentile
N0	10	12.5	22.5
N1	10	15	32.5
N2	7.5	12.5	22.5

Figure S7. The distributions and quartiles of durations of contact with tumor spheroids per neutrophil for N0, N1-like, and N2-like neutrophil subtypes. (A) Box plot showing the quartiles of each neutrophil subtype. **(B)** Table listing the values of the 25%, 50%, and 75% percentiles of each neutrophil subtype.

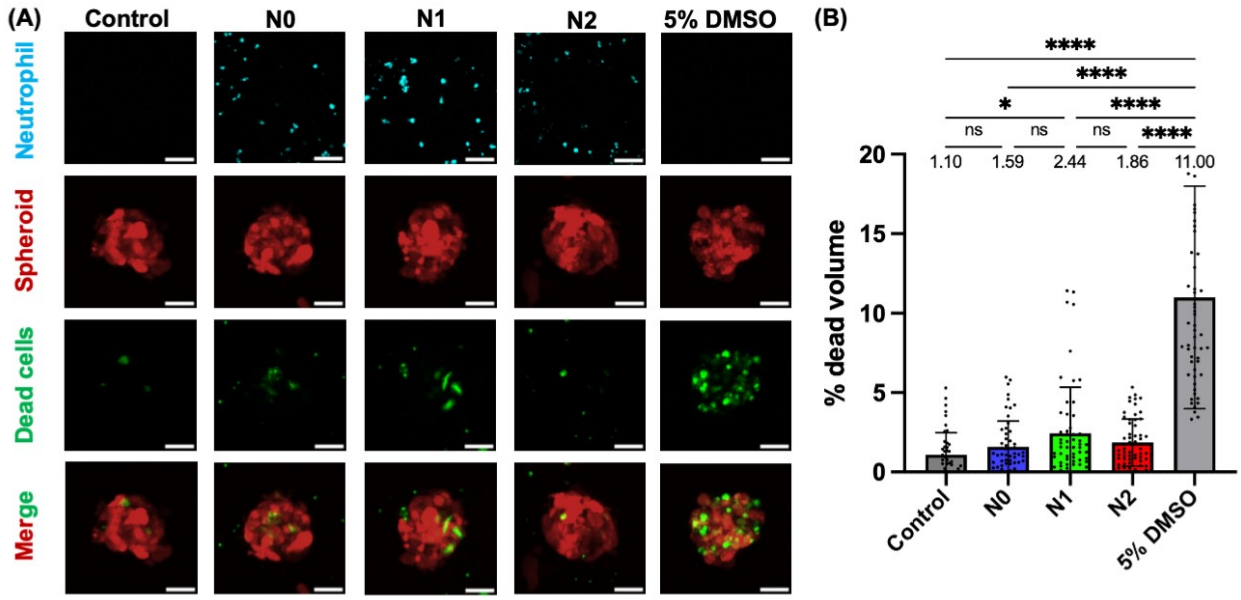


Figure S8. Both N1-like and N2-like neutrophils induced limited overall death of PANC-1 tumor spheroids. **(A)** Representative 10X confocal images showing the death (DRAQ7, green) of tumor spheroids (red) fixed after treatment with culture medium alone (control), N0 neutrophils, N1-like neutrophils, N2-like neutrophils, or 5% DMSO (positive control) for 24 h in the NTI-chip. Neutrophils (blue) were also shown. Images were maximum intensity projections of z-stacks with a 2 µm step size. Scale bar, 50 µm. **(B)** A bar plot showing the percentage of dead volume per tumor spheroid in specified conditions. Dead signals co-localized with neutrophil signals were excluded from the dead volume to remove dead neutrophils. Each data point represents a spheroid and n = 54-58 spheroids per condition. Bars show mean ± SD with mean values written above the points. Two independent experiments were performed. ns: $p \geq 0.05$, *: $p < 0.05$, ****: $p < 0.0001$, Kruskal-Wallis test.

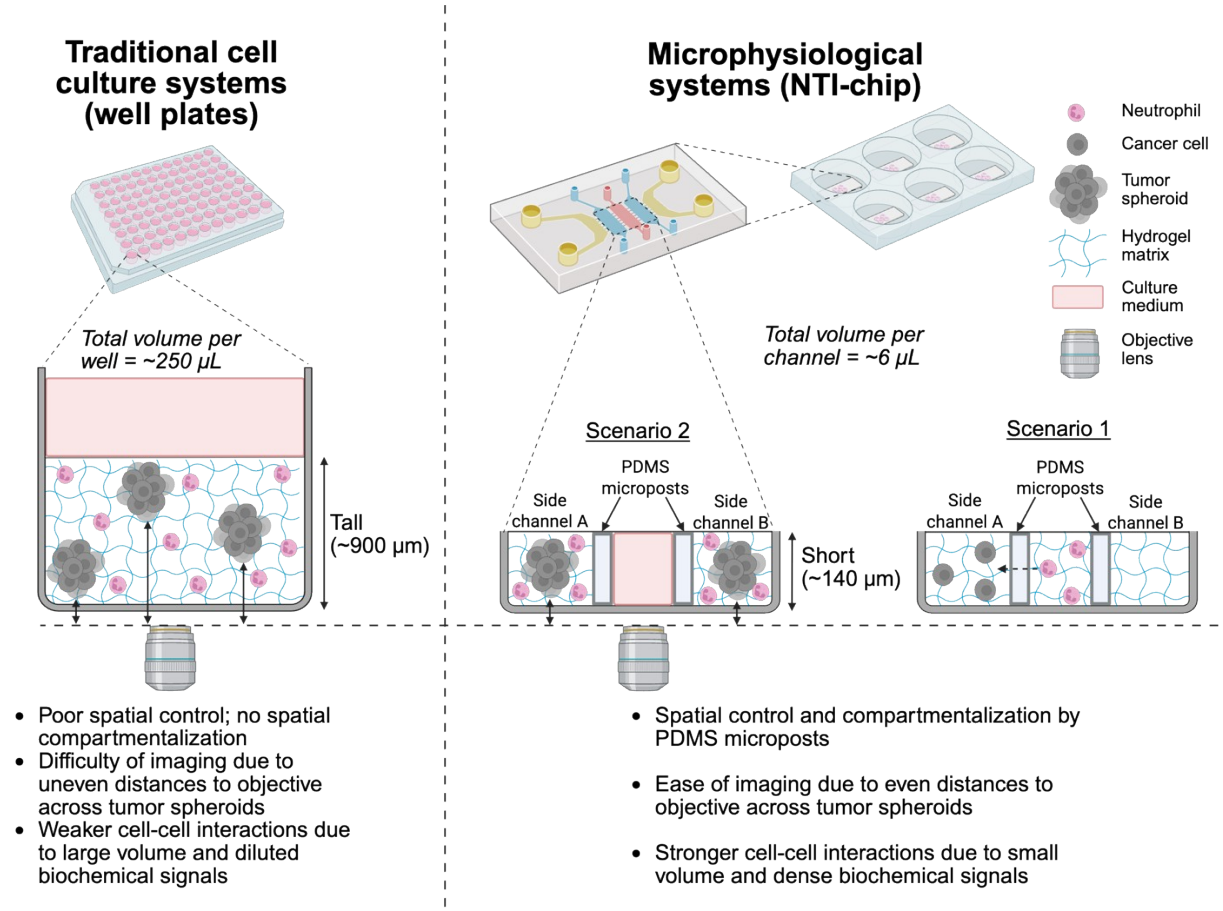


Figure S9. Advantages of using the microphysiological system NTI-chip to study neutrophil-cancer interactions over traditional well plate-based cell culture systems. The PDMS microposts in the NTI-chip enable compartmentalization and spatial control of neutrophils and cancer cells or tumor spheroids (loaded in the same channel or in two separate channels), a feature that is absent in well plates. In contrast to the tall thickness of the hydrogel (~900 µm) and thus uneven distances of different tumor spheroids to the objective lens of the microscope in well plates, the NTI-chip ensures an almost even distance of different tumor spheroids to the objective lens due to the small height of its microfluidic channels (~140 µm) relative to the diameter of the spheroid (~100 µm), which makes imaging faster and easier. Compared to large volumes in well plates (~250 µL for one well on a 96-well plate), the small volume of microfluidic channels in the NTI-chip (~6 µL) also leads to faster diffusion and higher concentrations of biochemical signals and thus stronger neutrophil-cancer interactions and elevated neutrophil behaviors to be measured in the study.

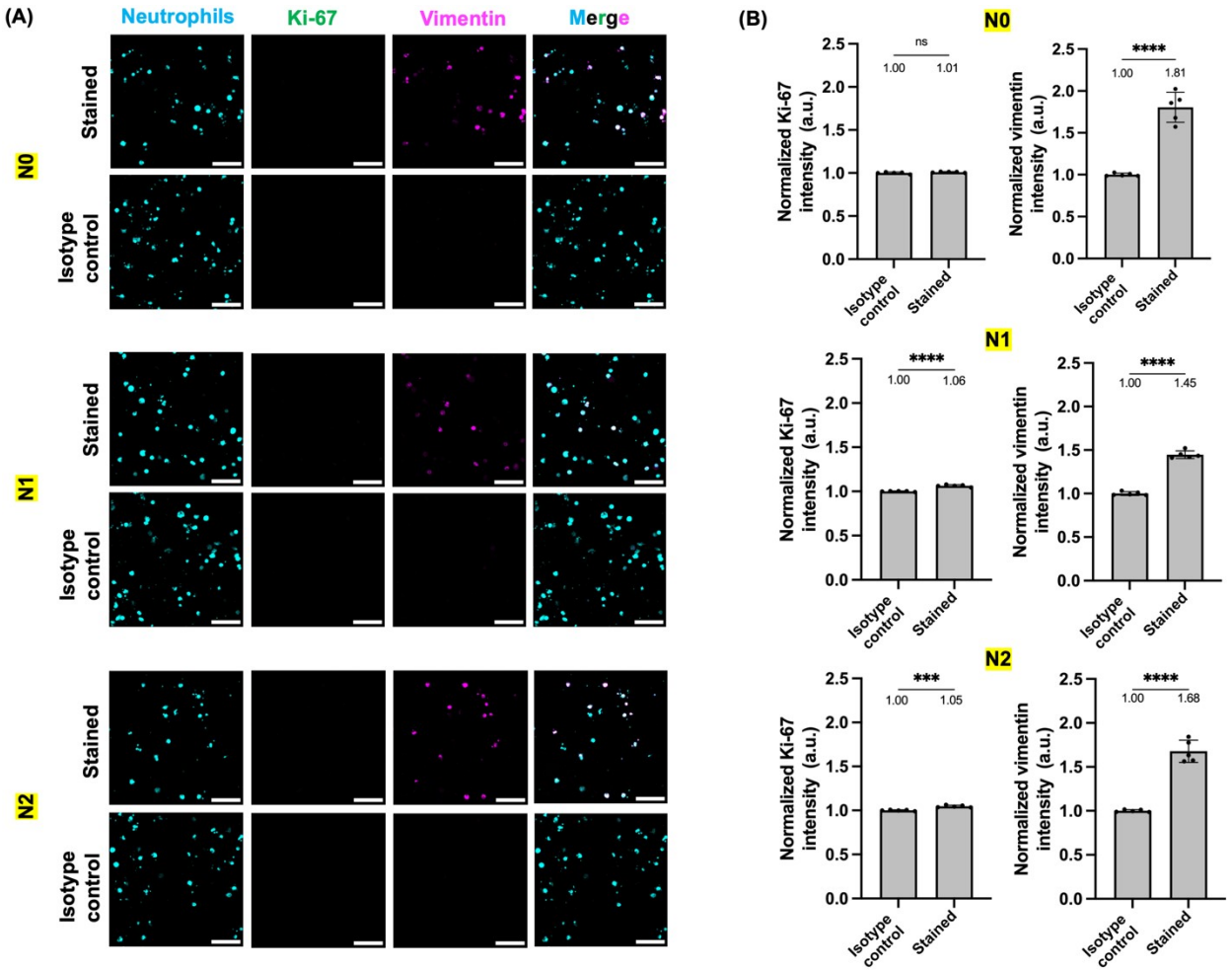


Figure S10. N0, N1-like, and N2-like neutrophils do not express Ki-67 but express vimentin. (A) Representative 20X confocal images of N0, N1-like, and N2-like neutrophils (blue) immunostained for Ki-67 (green) and vimentin (magenta) at t=24 h after being embedded in collagen hydrogel and loaded into the NTI-chip. Samples stained with the secondary antibody only were used as isotype control. Images are maximum intensity projections of z-stacks with a 2 µm step size. Scale bar, 50 µm. **(B)** Bar plots showing the normalized Ki-67 intensity (left panel) and vimentin intensity (right panel) of N0, N1-like, and N2-like neutrophils. Each data point represents the median value of all neutrophils per ROI. Raw values were normalized by the mean of the isotype control condition. n = 5 ROIs per condition. Bars show mean ± SD with the mean values written above the points. One experiment was performed. ns: $p \geq 0.05$, ***: $p<0.001$, ****: $p<0.0001$, unpaired t test.

Supplementary Tables

Marker	Neutrophil subtype	Experiment number					Mean	SD	SD/Mean (%)
		1	2	3	4	5			
CD11b (%)	N1	97.30	96.30	96.50	96.00	N/A	96.53	0.56	0.58
	N2	88.30	93.60	91.85	90.05	N/A	91.33	2.29	2.50
CD54	N1	13.97	13.67	14.78	N/A	N/A	14.14	0.57	4.06
	N2	1.84	1.89	2.06	N/A	N/A	1.93	0.12	5.98
CD62L	N1	0.50	0.53	0.50	N/A	N/A	0.51	0.02	3.40
	N2	0.96	0.94	0.94	N/A	N/A	0.94	0.01	1.23
CD182	N1	0.50	0.48	0.52	0.48	0.51	0.50	0.02	3.58
	N2	1.08	1.25	1.07	1.25	1.15	1.16	0.09	7.56

Table S1. Experiment-to-experiment variation in the surface marker expressions of N1-like and N1-like dHL-60 neutrophils. N1-like and N2-like neutrophils were prepared from the same batch of HL-60 cells at different passages. The surface expression levels of general neutrophil marker CD11b were measured as the percentage of CD11b-positive cells, and those of N1/N2-related markers CD54 (ICAM-1), CD62L (L-selectin), and CD182 (CXCR2) were measured as the normalized median fluorescence intensity (MFI). At least three independent experiments were repeated for the measurement of each marker. The value of each individual experiment, the mean value, the standard deviation (SD) among different experiments, and the SD divided by the mean are listed for each marker.

Supplementary Video Legends

Video S1. Representative video showing the migration of N0 neutrophils (blue) from the central channel into side channel A with cancer cells (red) and side channel B with empty hydrogel in the NTI-chip every 2 h over 24 h of time-lapse live imaging. Scale bar, 100 μm.

Video S2. Representative video showing the motility of N0 neutrophils (gray) after migration from the central channel into side channel A in the NTI-chip every 2 h over 8 h of time-lapse imaging and the extraction of single-neutrophil trajectories (color-coded by speed) using TrackMate (ImageJ). Neutrophils were tracked over 20-min intervals at each time point. Scale bar, 100 μm.

Video S3. Representative video showing N0 neutrophils (blue) interacting with PANC-1 tumor spheroids (brightfield) during 6 h of time-lapse imaging in the NTI-chip. The video is overlaid with neutrophil tracks extracted by TrackMate. Any neutrophils that entered the circular ROI (yellow) around the spheroid are considered to be in contact with the spheroid. For the particular tumor spheroid shown in this video, the frequency of contact with neutrophils is 6 and the mean duration of contact is 18.8 min. Scale bar, 50 μm.

Video S4. Representative video showing N1-like neutrophils (blue) interacting with PANC-1 tumor spheroids (brightfield) during 6 h of time-lapse imaging in the NTI-chip. The video is overlaid with neutrophil tracks extracted by TrackMate. Any neutrophils that entered the circular ROI (yellow) around the spheroid are considered to be in contact with the spheroid. For the particular tumor spheroid shown in this video, the frequency of contact with neutrophils is 12 and the mean duration of contact is 37.3 min. Scale bar, 50 μm.

Video S5. Representative video showing N2-like neutrophils (blue) interacting with PANC-1 tumor spheroids (brightfield) during 6 h of time-lapse imaging in the NTI-chip. The video is overlaid with neutrophil tracks extracted by TrackMate (ImageJ). Any neutrophils that entered the circular ROI (yellow) around the spheroid are considered to be in contact with the spheroid. For the particular tumor spheroid shown in this video, the frequency of contact with neutrophils is 9 and the mean duration of contact is 18.1 min. Scale bar, 50 μm.