Supplementary Material and Methods

Blood cell isolation

Heparinized peripheral blood samples were collected from healthy donor volunteers. Patient blood samples were collected from indicated types of patients after obtaining informed consent, and in accordance with the Declaration of Helsinki (version Seoul 2008).

Blood leukocytes were separated based on density by centrifugation over isotonic Percoll (Pharmacia, Uppsala, Sweden) with a specific density of 1.076 g/mL. The interphase fraction, containing peripheral blood mononuclear cells (PBMC), was harvested for isolation of untouched T cells by magnetic-activated cell sorting with the Pan T cell isolation kit of Miltenyi-Biotec (Bergisch Gladbach, Germany) according to the manufacturer's instructions. Neutrophils were obtained from the pellet fraction after erythrocyte lysis with hypotonic ammonium chloride solution at 4 °C.

ROS production

NADPH oxidase activity was measured by assaying the hydrogen peroxide production by neutrophils in response to various stimuli with the Amplex Red kit (Molecular Probes, Life Technologies, Carlsbad, CA, USA). Neutrophils ($1x10^6$/mL) were stimulated in Hepes-buffered saline solution with fMLF (1 µM), TNFα (10 ng/mL), C5a (10^{-2} µM), IL-8 (84 ng/mL), PAF (1 µM), LPS (20 ng/mL) + LPS-binding protein (LBP) (50 ng/mL, R&D Systems, Minneapolis, MN, USA) in the presence of Amplex Red (0.5 µM) and horseradish peroxidase (1 U/mL). Fluorescence was measured at 30-second intervals during 4 hours with the HTS7000+ plate reader (Tecan, Zurich, Switzerland). Maximal slope of hydrogen peroxide release was assessed over a 2-minute interval.

Degranulation

Release of primary/azurophilic and secondary/specific granules after stimulation were confirmed by performing myeloperoxidase (MPO) and lactoferrin ELISA (Hycult Biotech, Plymouth Meeting, PA,

USA), respectively, on cell supernatants harvested after 4 hours of stimulation at 37 °C, according to the manufacturer's instructions.

Apoptosis assessment

Purified T cells and neutrophils (1:3 ratio) were cultured in the presence of anti-CD3/anti-CD28 antibodies, IL-15, or PHA and in the presence/absence of TNFα. When indicated, pan-caspase inhibitor zVAD (Abcam, 100 μM) was added to the culture to investigate further the role of apoptosis. After 2 days of co-culturing, T cells were harvested and stained with annexin-V-FITC (BD Biosciences) and Hoechst (Sigma-Aldrich). Alternatively, cells were fixed and permeabilized for intracellular staining with an antibody recognizing active caspase-3 (clone C92-605, BD Biosciences) or an isotype control antibody (clone 3452, Cell Signaling Technology). Staining's were quantified by flow cytometry.

Live cell imaging

Purified T cells were labelled with Vybrant DiD cell-labeling solution (Molecular Probes) and co-cultured with neutrophils (1:3 ratio) in the presence of anti-CD3/anti-CD28 antibodies, TNFα (10 ng/mL), and DHR (Molecular Probes, 250 μM) on a Permanox chamber slide (Thermo Scientific). The co-culture was imaged for 4 hours (20 second intervals) with a Leica SP8 Confocal Laser Microscope.

For live cell imaging of the mitochondria, purified T cells were FACS sorted after 2 day co-culture in presence of IL-15 and TNFα-activated neutrophils to generate small and large T cells. Cells were stained with Mitotracker Green (Invitrogen, Carlsbad, CA, USA) and Hoechst in IMDM (Gibco Life Technologies) supplemented with 5% HSA for 30 minutes at 37 °C in the dark, followed by washing with IMDM+5%HSA and incubation on coverslips for 30 minutes at 37 °C. Cells were imaged with a Leica SP8 Confocal Laser Microscope.

Measuring uptake of T cell membrane by neutrophils

To measure the membrane uptake by flow cytometry, purified T cells were also labelled with Vybrant DiD cell-labeling solution and co-cultured with neutrophils (1:3) ratio in the presence/absence of IL-15, fMLF (1 µM), LPS (20 ng/mL), or TNFα (10 ng/mL) and with or without a CD11b-blocking antibody or isotype control. After 3 hours, cells were harvested and the number and percentage of DiD-positive neutrophils were determined by flow cytometry.

Electron Microscopy

After 2 days of coculturing purified T cells and neutrophils (1:3 ratio) in the presence of IL-15 and TNFα, cells were harvested and physically separated into small and large T cells by FACS sorting based on FSC/SSC. For EM imaging, small and large T cells, with addition of erythrocytes due to a low yield, were fixed in Karnovsky's fixative. Post fixation was done with 1% (w/v) osmiumtetroxide in 0.1 M cacodylatebuffer. After washing, the cells were stained en bloc with Ultrastain 1 (Leica, Vienna, Austria), followed by ethanol dehydration series. Finally, the samples were embedded in a mixture of DDSA/NMA/Embed-812 (EMS, Hatfield, PA, USA), sectioned and stained with Ultrastain 2 (Leica, Vienna, Austria) and analyzed with a Tecnai12G2 electron microscope (FEI, Eindhoven, The Netherlands).

Sample preparation for mass spectrometry analysis

Tryptic peptides were prepared according to the method described by Kulak *et al.*(39) with some adjustments. Briefly, cells were lysed in 30 µL of 1% sodium deoxy cholate (Sigma Aldrich) 10 mM TCEP (Thermo Scientific), 40 mM chloroacetamide (Sigma Aldrich), 100 mM TRIS-HCl pH 8.0 (Life Technologies, Paisly, UK) and HALT protease/phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA), incubated at 95 °C for 5 minutes and sonicated for 10 minutes in a Sonifier bath (Branson, Dansbury, CT, USA). An equal volume of 50 mM ammonium bicarbonate (Sigma Aldrich), pH 8.0 was added, containing 200 ng trypsin Gold (Promega, USA) was present. Proteins were digested overnight at room temperature. Subsequently, samples were acidified by addition of 1 µL

trifluoroacetic acid (Thermo Scientific) and loaded on in-house prepared SDB-RPS STAGEtips (Empore, St. Paul, MN, USA). Peptides were desalted and eluted in 5% (v/v) ammonium hydroxide (Sigma Aldrich), 80% (v/v) acetonitrile (BioSolve, Valkenswaard, The Netherlands). Sample volume was reduced by SpeedVac and supplemented with 2% acetonitrile, 0.1% trifluoro acetic acid (TFA) to a final volume of 10 µL. 3 µL of each sample was injected for MS analysis.

Mass spectrometry data acquisition

Tryptic peptides were separated by nanoscale C18 reverse-phase chromatography coupled on line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) via a nanoelectrospray ion source (Nanospray Flex Ion Source, Thermo Scientific). Peptides were loaded on a 20 cm 75–360 µm inner-outer diameter fused silica emitter (New Objective, Woburn, MA, USA) packed in-house with ReproSil-Pur C18-AQ, 1.9-µm resin (Dr Maisch GmbH, Ammerbuch, Germany). The column was installed on a Dionex Ultimate3000 RSLC nanoSystem (Thermo Scientific) with a MicroTee union formatted for 360 µm outer diameter columns (IDEX) and a liquid junction. The spray voltage was set to 2.15 kV. Buffer A was composed of 0.5 % acetic acid and buffer B of 0.5 % acetic acid, 80% acetonitrile. Peptides were loaded for 17 min at 300 nl/min in 5% buffer B, equilibrated for 5 minutes at 5% buffer B (17-22 min) and eluted by increasing buffer B from 5-15% (22-87 min) and 15-38% (87-147 min), followed by a 10-minute wash to 90 % and a 5-minute regeneration to 5%. Survey scans of peptide precursors from 400 to 1500 m/z were performed at 120K resolution (at 200 m/z) with a 1.5×10^5 ion count target. Tandem mass spectrometry (MS^2) was performed by isolation with the quadrupole with isolation window 1.6, Higher-energy Collison induced Dissociation (HCD) fragmentation with normalized collision energy of 30, and rapid scan mass spectrometry analysis in the ion trap. The MS^2 ion count target was set to 1.5×10^4 and the max injection time was 35 ms. Only those precursors with charge state 2–7 were sampled for MS^2. The dynamic exclusion duration was set to 60 s with a 10-ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3 s cycles. All data were acquired with Xcalibur software.

Mass spectrometry data analysis

The RAW mass spectrometry files were processed with the MaxQuant computational platform(40), 1.5.3.30. Proteins and peptides were identified with the Andromeda search engine by querying the Human Uniprot database (downloaded March 2017). Standard settings with the additional options match between runs, Label Free Quantification (LFQ), and unique peptides for quantification were selected. The generated 'proteingroups.txt' table was filtered for potential contaminants, reverse hits and 'only identified by site' with Perseus 1.6.0.2. The LFQ values were transformed in log2 scale, the replicates per experimental condition grouped and averaged based on the median, and proteins were filtered for at least two valid values in one of the experimental groups. Missing values were imputed by normal distribution (width=0.3, shift = 1.8), assuming these proteins were close to the detection limit. Quantitative significance (Two-sample T-tests (s0=2) and Principal Component Analysis) and MitoCarta2.0(41) annotation were performed using Perseus software. GO-term enrichments were performed in Cytoscape 3.6.0(42) using the BiNGO 3.0.3(43), EnrichmentMap 3.0.0(44) and stringapp 1.2.2(45) plugins. The .raw MS files and search/identification files obtained with MaxQuant have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org/cgi/GetDataset) via the PRIDE partner repository(46) with the dataset identifier PXD010508.

Flow cytometry and FACS sorting

For characterization of the surface expression of various markers on small and normal-sized T cells, the following antibodies were used: PerCPCy5.5-labeled anti-TCRαβ (clone 1IP26, Biolegend), FITC-labeled anti-CD3 (clone UCHT1, Invitrogen), APC-labeled anti-CD4 (clone SK3, BD Biosciences), PerCPCy5.5-labeled anti-CD8 (clone SK1, Biolegend), PE-labeled anti-CD25 (clone 2A3, BD Biosciences), APC-labeled anti-CD62L (clone DREG-56, BD Biosciences), FITC-labeled anti-CD11a (clone CLB-LFA-1/2, TB 133,

Sanquin Reagents, Amsterdam, The Netherlands), FITC-labeled anti-CD18 (clone MEM48, Diaclone, Besançon cedex, France), FITC-labeled anti-CCR6 (clone 53103, R&D Systems).

For characterization of the surface expression on low-density and high-density neutrophils, the following antibodies were used: PB-labeled anti-CD11b (clone ICRF44, BD Biosciences), PECy7-labeled anti-CD16 (clone 3G8, BD Biosciences), FITC-labeled anti-EMR3 (clone 3D7, Bio-Rad, Hercules, CA, USA), PE-labeled anti-siglec8 (clone 7C9, Biolegend) and PE-labeled anti-LOX1 (clone 15C4, Biolegend).

Neutrophils from healthy donors were left unstimulated or were primed with CytoB or PAF for 5 minutes at 37°C and subsequently stimulated with fMLF for 10 minutes at 37°C. For characterization of the surface expression on the unstimulated or stimulated neutrophils, the following antibodies were used: PE-labeled anti-LOX1, FITC-labeled anti-CD66b (clone CLB-B13.9, Pelicluster Sanquin, Amsterdam, The Netherlands) and APC-labeled anti-CD15 (clone MMA, Thermo Scientific).

Flow cytometry data were acquired using Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, USA).

Small and large T cells were separated by FACS sorting based on either FSC/SSC and CFSE positivity of the T cells or based on FSC/SSC and FITC-labeled anti-CD15 (clone MMA, BD biosciences) or APC-labeled anti-CD15 negativity, to exclude neutrophils from the sorted fractions. FACS sorting was performed using BD FACS Aria III (BD biosciences).

Analysis of ATP levels

Unlabeled purified T cells were FACS sorted after 2 days of culture where T cells were left unstimulated or cultured in presence of IL-15 and TNFα with or without neutrophils. ATP levels were determined in neutralized perchloric acid extracts of the indicated T cell populations. In short, extracts were made by adding 500 µL phosphate-buffered saline to the cell pellet, between 1.5-4x10^6 cells, after 2 minute centrifugation the cell pellet was acidified with 150 µL of perchloric acid (2.4%wt/ vol). After 15 min on ice, extracts were centrifuged at 4 °C at 6000 x g and 7.5 µL of 5 mol/L K2 CO3 was added to 150 µL deproteinized supernatant for neutralization. Samples were kept frozen until analysis. Adenine

nucleotides ATP, ADP and AMP were assayed using a HPLC method(47). The measured concentrations were divided by the cell count. Values for adenine nucleotides are thus expressed in pmol/10^6 cells. Total adenylate was calculated as the sum of ATP, ADP and AMP levels.

Statistics

Statistical analysis was performed with GraphPad Prism version 7 for Windows (GraphPad Software, San Diego, CA, USA). Data were evaluated by one-way ANOVA or unpaired two-tailed student's *t*-test. The results are presented as the mean ± SEM. Data were considered significant when $p<0.05$.

Supplementary figures

Suppl. Figure 1 *– Activated neutrophils suppress cytokine production.*

Supernatants of cultures (purified T cells in the presence/absence of anti-CD3 antibody, anti-CD28 antibody with unstimulated or fMLF-activated neutrophils) were harvested after 4-6 days and analyzed by Luminex for the presence of cytokines IFNγ (A), IL-13 (B), and IL-17 (C) (mean; n=3).

Suppl. Figure 2 *– Mixing neutrophils and T cells from different donors does not induce alloreactivity.*

Purified T cells were cultured in the presence/absence of anti-CD3 antibody, anti-CD28 antibody with unstimulated or fMLF-activated neutrophils derived from the same donor or from another healthy control individual. Cells were harvested after 5-6 days and analyzed by flow cytometry for CFSE dilution among $CD4^{POS}$ (A) and $CD8^{POS}$ T cells (B). Error bars indicate SEM, **** p<0.0001, *** p<0.001, n=5.

Suppl. Figure 3 *– Surface marker expression of low-density and high-density neutrophils from head-neck cancer patients and healthy controls.*

A) Low-density and high-density neutrophils from head-neck cancer patients (HNC, black circle), mammacarcinoma patients (MC, black triangle) and healthy controls (grey square) were analysed by flow cytometry for granulocyte-associated (CD11b, CD16, EMR3, Siglec8) or MDSC-associated (LOX-1) surface markers. Shown is the percentage of positive cells (upper graphs) or the MFI (lower graphs) of the indicated surface markers. n=3-8 **B)** Expression of surface markers LOX-1, CD66b and CD15 of unstimulated (light grey), PAF/fMLF-stimulated (dark grey) or CytoB/fMLF (blue) stimulated neutrophils from healthy donors. Shown are representative FACS plots of n=5. Error bars indicate SEM, * p<0.05.

Suppl. Figure 4 *– Small T cells are not positive for apoptotic markers.*

A) T cells cultured for 2 days in the presence/absence of neutrophils, TNFα, or one of the indicated T cell stimulating agents and were analyzed by flow cytometry for Annexin-V and Hoechst binding. Shown are the percentages of each population: $Hoechst^{POS}/AnnV^{NEG}$, late apoptotic/Dead ($Hoechst^{pos}/AnnV^{POS}$), early apoptotic ($Hoechst^{NEG}/AnnV^{POS}$) and live population ($Hoechst^{NEG}/AnnV^{NEG}$) (n=3). **B)** Flow cytometric analysis of cytoplasmic presence of cleaved caspase-3 in T cells after indicated culture conditions of 2 days, in presence or absence of pan-caspase inhibitor zVAD (100 µM). Shown are representative FACS plot (n=3). Error bars indicate SEM, **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05. **C-D)** Purified CFSE labeled T cells from control donors were cultured with anti-CD3 and anti-CD28 antibodies (white bars), and in the presence of neutrophils (black bars) and/or pan-caspase inhibitor zVAD (**C**, 100 µM) or RIPK1 kinase inhibitor necrostatin-1 (**D**, 100 µM). Cells were harvested after 5 days and analyzed by flow cytometry for CFSE dilution among the total T cell population (n=4 donors tested in duplicate). Error bars indicate SEM; **** p<0.0001, *** p<0.001, ** p<0.01, * p<0.05.

Suppl. Figure 5 – *Proteomic comparison of small and large T cells.*

T cells cultured for 2 days in the presence of IL-15 and TNFα-activated neutrophils were sorted into small and large T cells for proteomic comparison **A)** Gene Ontology (GO) term enrichment analysis of proteins increased (red) or decreased (blue) in small T cells. **B)** Protein-protein interactions of affected proteins as evaluated by STRING analysis. The node size reflects the LFQ intensity difference and the color whether the protein abundance is increased (red) or decreased (blue) in small T cells compared to large T cells.

Suppl. Figure 6 – *EM images of small T cells.*

Representative EM images of small T cells after 2 days of culture with IL-15 and TNFα-activated neutrophils. Depicted are (auto) phagolysosomes (A), dilated endoplasmic reticulum (ER), where the

perinuclear membrane is part of the ER (B) and swelling of mitochondria (C) emphasizing the poor condition of small T cells.

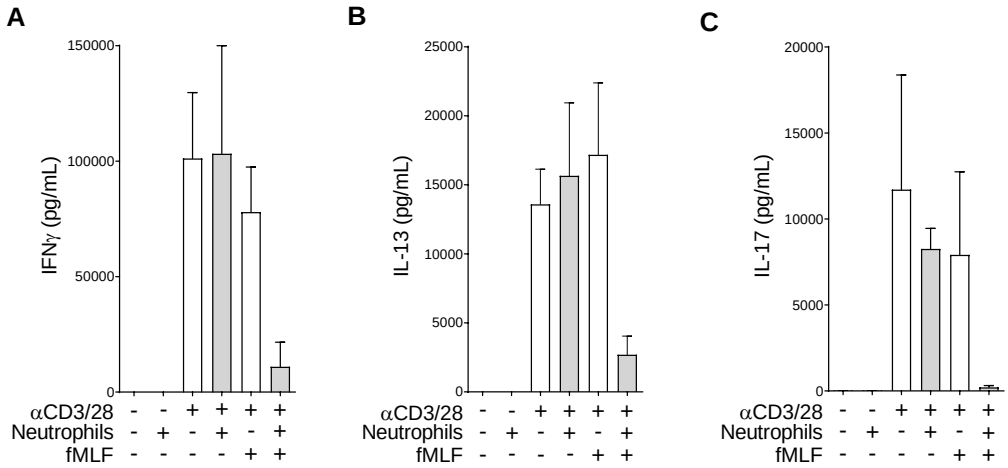
Suppl. Figure 7 – *Schematic model of MDSC activity of mature activated neutrophils.*

We describe the multifaceted mechanism in humans by which activated mature neutrophils suppress T cell proliferation. In a CD11b-dependent interaction with T cells, the combined process of trogocytosis, ROS formation, and degranulation results in non-apoptotic T cell paralysis. In the resulting small T cells, neutrophil-derived substances may act as the effector molecules to convey strong T cell suppression by metabolic stress but without complete T cell disintegration.

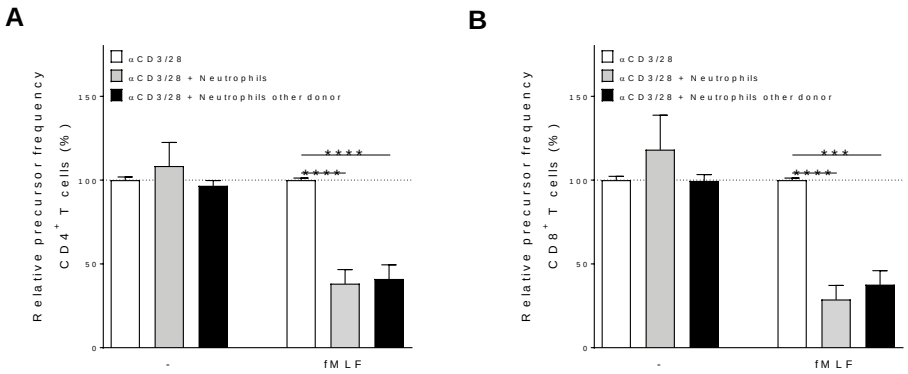
Suppl. Table 1 – *Differently abundant proteins in the small and large T cell populations.*

Results of a two-sample T-test as represented in the volcano plot of figure 6C. Shown are the proteins more (red dots in figure 6C) and less abundant (blue dots in figure 6C) in small T cells compared to large T cells.

Supplementary figure 1



Supplementary figure 2



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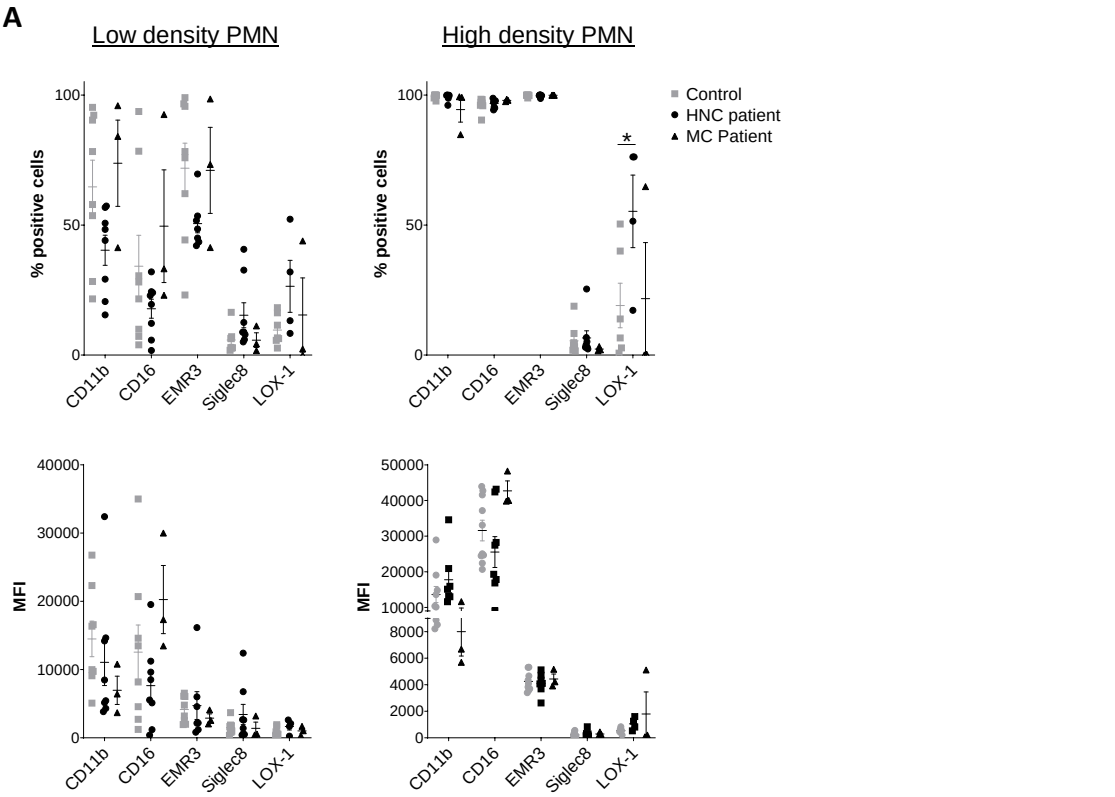
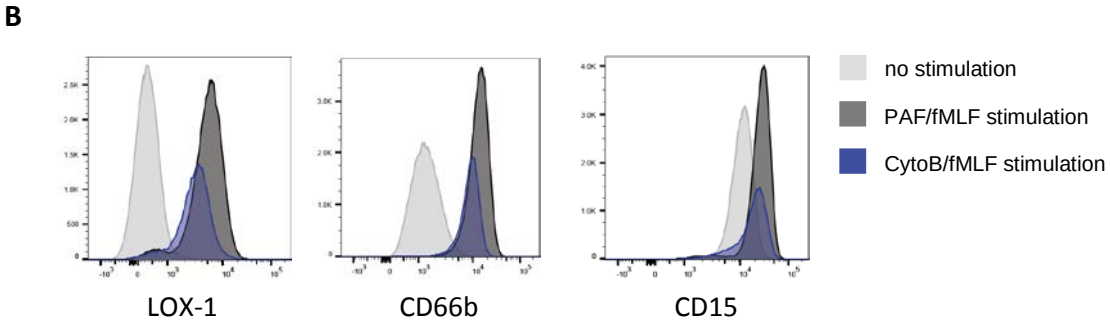
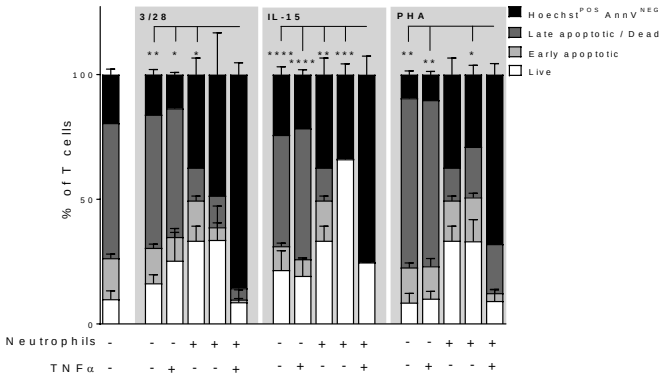




Supplementary figure 4

A



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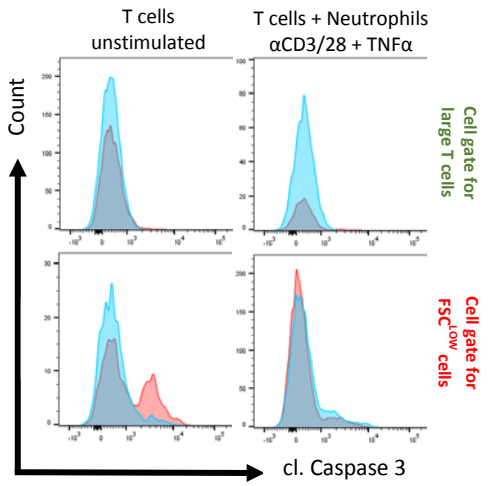
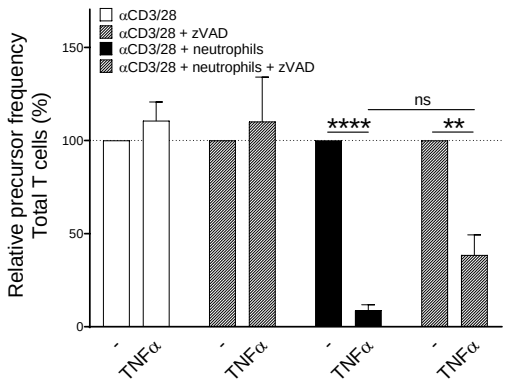


Red = without zVAD
Blue = with zVAD

C



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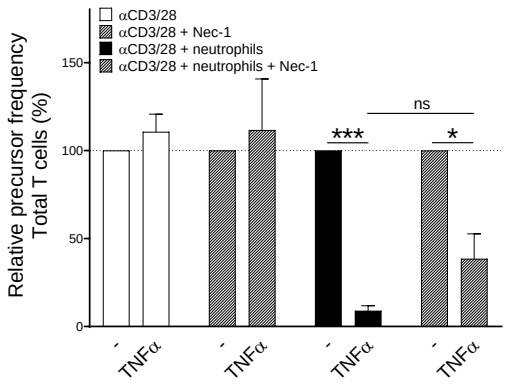
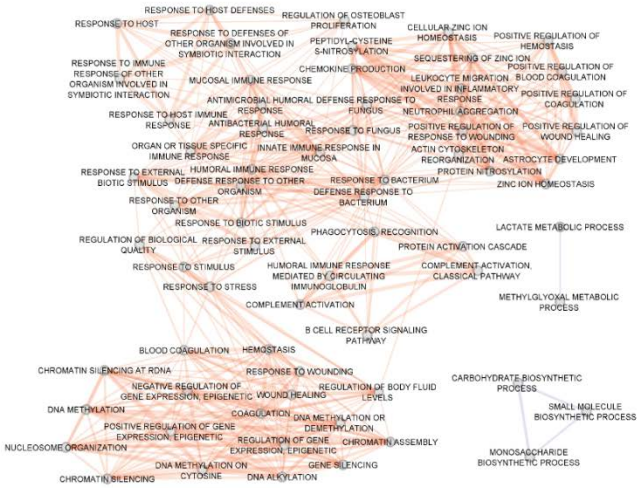


Supplementary figure 5

A



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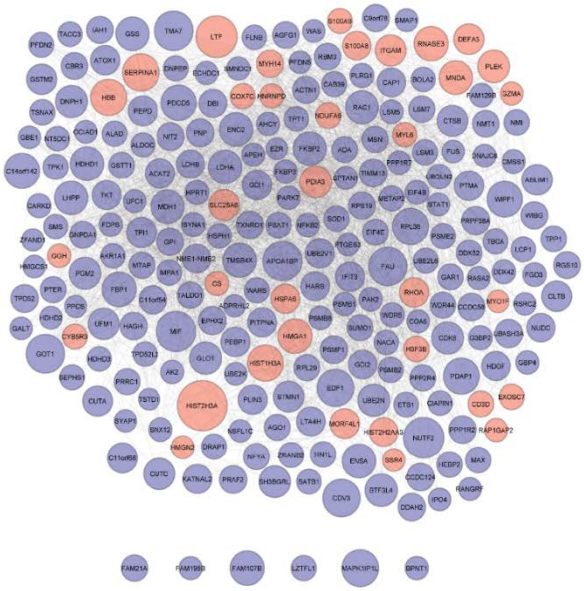
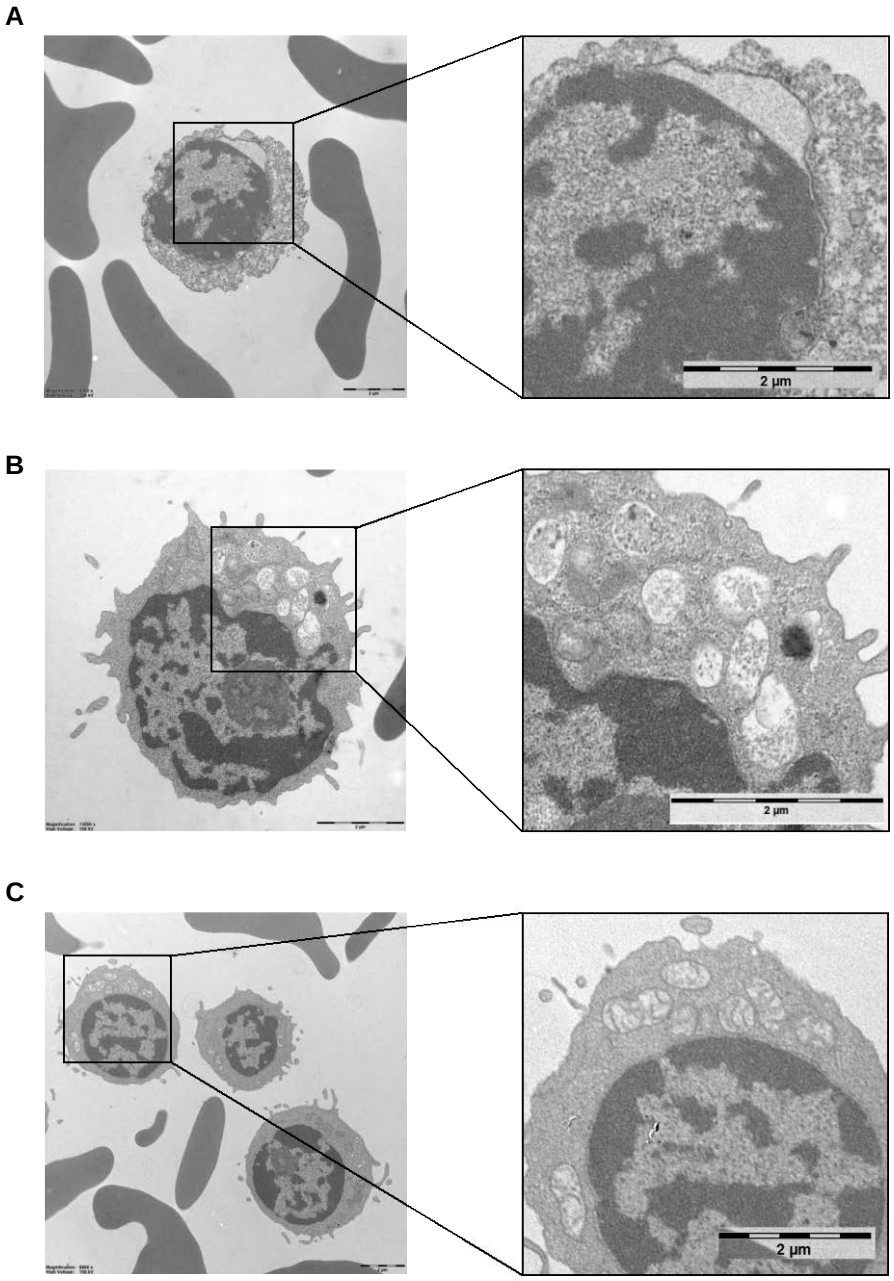


Supplementary figure 6



Supplementary figure 7