Supplemental Information

Methods

Cytology Slide Preparation, Staining, and Review

An Advia 120 or Advia 2120 Hematology System (Siemens) provided an automated WBC count on ascites from patients with newly diagnosed metastatic high-grade serous ovarian cancer (HGSOC), the most common histologic subtype of EOC. Cytospins were prepared using Cytopro slides and a Cytopro 760 Cytospin Centrifuge (Wescor). Slides were manually stained with Wright-Giemsa for morphologic evaluation. A hematopathologist (JTW) performed morphologic analysis of the slides. Differential counts (300 total cell counted) of the inflammatory cells in the ascites were performed. Tumor cells and mesothelial cells were excluded from the differential count.

Antibodies and Staining of Peripheral Blood and Ascites

Cells were subjected to RBC lysis, followed by washing and staining in buffer (DPBS, 1% BSA, 2 mM EDTA). Fc receptors were blocked to prevent non-specific antibody binding prior to staining (15 min, 4°C; anti-mouse CD16/CD32, clone 2.4G2; BD Biosciences). Antibodies targeted to human CD45 (clone HI30), CD33 (P67.6), CD11b (CBRM1/5), CD15 (W6D3), CD14 (M5E2), and HLA-DR (L243) (Biolegend, San Diego, CA) were used to evaluate the proportion of granulocyte and monocyte/macrophage populations.

Coculture of PMN with Ascites and T Cells

Freshly isolated T cells (1e5) were stimulated with anti-CD3/CD28 Dynabeads (2.5 µl; Thermo Fisher Scientific) and cocultured with autologous PMN (1:1) and ascites supernatants (50% final well volume) in an incubator at 37°C, 5% CO_2. After 72h, [^{3}H] thymidine (1 µCi per well) was added and allowed to incorporate for 16-18h. Cells were harvested onto a Filtermat and counted on a Beta counter. Results are expressed as net cpm [calculated by subtracting the average cpm of unstimulated T cells from the average cpm of stimulated T cells].

Where indicated, cells were pelleted for immunophenotyping and supernatants were saved for ELISA at time points throughout coculture. Viability was assessed by staining with Annexin V and/or PI (Dead Cell Apoptosis Kit, V13241, Thermo Fisher Scientific), following the manufacturer's protocols. For surface phenotyping and intracellular staining, cells were stained in buffer (DPBS, 1% BSA, 2 mM EDTA) and Cytofix/Cytoperm buffer kit (BD Biosciences), respectively, following the manufacturer's protocols. Data were analyzed using FCS Express 6.

Pretreatments of T cells, PMN, and Ascites Supernatants

Where indicated, T cells, PMN, and ascites supernatants underwent various pretreatments. Where indicated, T cells were pretreated for 1h with neutralizing antibody against ICAM-I (1-10 µg), IgG1 isotype (1-10 µg), or media (RPMI 1640, 5-10% heat-inactivated FBS, HEPES, sodium pyruvate, non-essential amino acids, and penicillin-streptomycin).

Where indicated, PMN were pretreated for 1h with neutralizing antibody against CD11b (1-10 µg), TGF-beta receptor 1 (1-10 µg) or IgG1 isotype (1-10 µg); thapsigargin (THG, 0.5-2 µM), diphenyleneiodonium (DPI, 1-25 µM), N-Formylmethionine-leucyl-phenylalanine (fMLF, 1-100 nM), brefeldin-A (1-10 µg/mL), ER export inhibitor 1 (Exo1, 20-75 µM), Cl-amidine (10-20 µM), SNARE decoys: TAT-SNAP23 (0.6-0.9 µg/mL), TAT-SYN4 (0.6-0.9 µg/mL), TAT-GST (0.6-0.9 µg/mL) (produced as described in the laboratory of Dr. Kenneth McLeish), puromycin and actinomycin-D (1-5 µg/mL), or with media.

Where indicated, ascites supernatants were pretreated with heat (56°C, 1h) to denature heat-labile constituents or proteinase-K (100 µM, 37°C, 12h) to degrade proteins. Two formulations of compstatin, a peptide C3 inhibitor, were used: CS (250 µM, 30°C, 2h, Tocris) and Cp40 (1.25-20 µM, 30°C, 2h), as well as a scramble peptide, provided by Dr. John Lambris (University of Pennsylvania). In separate studies, ascites supernatants were ultra-centrifuged (100,000g, 4°C, 1.5h) to separate the membrane-rich (MR-ASC) and membrane-depleted (MD-ASC) fractions. In others, ascites supernatants were pretreated for 1h with neutralizing antibody

against C5 (0.5-1.0 µg, A217; Quidel, San Diego, CA) or C7 (0.5-1.0 µg, A221; Quidel) or IgG1 isotype (0.5-1.0 µg), or with OmCI (0.6-1.2 µM), a peptide C5 inhibitor derived from the saliva of *Ornithodoros moubata* (35, 36), provided by Dr. Anna Blom (Lund University).

Where indicated, L-arginine (50 µM-1 mM), N-acetylcysteine (10-25 mM), DNase I (50-100 IU), rIL-2 (100 IU), Zileuton (50 µM), Indomethacin (10 µM), or 1-methyl-DL-tryptophan (1-MT; 100 µM) was added to the cocultures.

Antibodies and Staining of T Cells After Coculture

Fc receptors were blocked to prevent non-specific antibody binding prior to staining (15 min, 4°C; anti-mouse CD16/CD32, clone 2.4G2; BD Biosciences). For phenotyping of PMN, antibodies targeted to human CD45 (clone REA747), CD15 (VIMC6) (Miltenyi Biotec, Inc.), CD63 (H5C6), CD66b (G10F5), and CD35 (E11) (Biolegend) were used. For phenotyping of T cells, antibodies targeted to human CD45 (clone REA747), CD3 (REA613), CD4 (REA623), CD8 (REA734), CD62L (145/15), CD69 (REA824), CD40L/CD154 (REA238), CD107a (REA792), PD-1/CD279 (PD1.3.1.3), LAG-3/CD223 (REA351), CTLA-4/CD152 (REA1003) (Miltenyi Biotec, Inc.), IFN-gamma (4S.B3) (Biolegend), T-bet (4B10), Eomes (WD1928), and CCR7/CD197 (4B12) (Thermo Fisher Scientific) were used.

Sorting of T cell Populations

Donor T cells ($CD3^+$) isolated form peripheral blood were sorted (FACScan, Becton Dickinson) to isolate naïve ($CD3^+CD45RA^+RO^-CD62L^+$), central memory ($CD3^+CD45RA^-RO^+CD62L^+$), and effector memory ($CD3^+CD45RA^-RO^+CD62L^-$) populations. Post-sort analysis using antibodies targeted to human CD3 (clone REA613), CD45RA (REA562), CD45RO (REA611), CD62L (145/15) (Miltenyi Biotec, Inc) showed >90% purity. Forward scatter versus side scatter gating was set to include all non-aggregated cells.

Measurement of Interleukin-2 by ELISA

Interleukin-2 (IL-2) levels from banked coculture supernatants were measured by Quantikine ELISA for Human IL-2, according to manufacturer's protocol (D2050, R&D Systems).

Preparation of PMN for Proteomics Analysis

PMN were isolated from peripheral blood, as previously described. Ascites supernatants were pretreated with proteinase-K (100 µM, 37°C, 12h). PMN (5e6; 5 technical replicates) were exposed to untreated ascites supernatants, proteinase-K-digested ascites supernatants, or media (RPMI 1640, 5-10% heat-inactivated FBS, HEPES, sodium pyruvate, non-essential amino acids, and penicillin-streptomycin) for 30 or 60 min. PMN were washed, decanted of all liquid, flash frozen on dry ice, and stored at -80°C for subsequent proteomic analysis.

PMN (5e6) were resuspended in 1 mL surfactant cocktail buffer containing 50 mM Tris-formic acid (FA; pH 8.0) containing 150 mM NaCl, 2% sodium dodecyl sulfate (SDS), 0.5% SDC, and 2% IGEPAL CA-630, with complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Samples were placed on ice for 10 min, and then sonicated with 5 sonication-chill cycles until the liquid became pellucid. The sonicated samples were placed on ice for another 30 min, and then centrifuged (20,000g, 30 min, 4°C). Supernatants were carefully transferred to new low-bind tubes, and the protein concentration of all samples was determined by bicinchoninic acid assay (BCA; Pierce Biotechnology, Inc., Rockford, IL).

For each sample, 100 µg of extracted proteins (normalized to 1 µg/µL by 0.5% SDS) were used for LC-MS analysis. Proteins were first reduced with 10 mM dithiothreitol (DTT; 30 min, 56°C) and alkylated using 20 mM iodoacetamide (IAM; 30 min, 37°C). Both steps were performed while covered with aluminum foil with constant agitation. Denatured proteins were precipitated by the addition of 7 volumes of chilled acetone, followed by incubation (3h, -20°C). After centrifugation (20,000g, 30 min, 4°C), the pelleted proteins were washed with 500 µL methanol, briefly air-dried, and resuspended in 80 µL 50 mM Tris- FA. A total of 5 µg trypsin (Sigma-Aldrich, St. Louis, MO) dissolved in 20 µL 50 mM Tris-FA was added to the protein

pellets at a final enzyme:substrate (E:S) ratio of 1:20, and the proteins were incubated (18h, 37°C) with constant vortexing. Derived peptides were acidified by adding 1% FA, centrifuged (20,000g, 30 min, 4°C) and transferred to LC vials.

Proteomics LC-MS Analysis

RPLC separation of derived peptides was performed on a Dionex Ultimate 3000 nano LC system and an Ultimate 3000 gradient micro LC system with a WPS-3000 autosampler (Thermo Fisher Scientific, San Jose, CA). Mobile phase A and B were 0.1% formic acid in 2% acetonitrile and 0.1% FA in 88% acetonitrile. A total of 4 µg peptides were loaded onto an RP trap (300 µm ID x1 cm), with 1% mobile phase B at a flow rate of 10 µL/min, and the trap was washed for 3 min. A series of nano-flow gradients (flow rate at 250 nL/min) was set to back-flush the trapped samples onto the nano-LC column (75-µm ID x 100 cm), which was heated at 52°C to improve chromatographic resolution and reproducibility. The optimized gradient profile was 4-13% B for 15 min; 13-28% B for 110 min; 28-44% B for 5 min; 44-60% B for 5 min; 60-97% B for 1 min, and isocratic at 97% B for 17 min. Peptides eluted from nano-LC was analyzed by an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, San Jose, CA). MS was operated under the data dependent mode. MS1 spectra were collected at 120,000 resolution with an automated gain control (AGC) target of 500,000 and a max injection time of 50 ms. Previously interrogated precursors were excluded using a dynamic window (60s ± 10 ppm). For MS2, precursor isolation window was set to 1 Th, and precursor ions were fragmented by high-energy collision dissociation (HCD) at a normalized collision energy of 35%. MS2 spectra were collected at 15,000 resolution with an AGC target of 50,000 and a max injection time of 50 ms.

LC-MS raw files were processed with an in-house developed MS1-based label-free quantification pipeline, IonStar. Peptide identification was performed by MS-GF+ (v9979, released on 03/26/2014) searching against a Uniprot-Swissprot Homo sapiens protein database (20200 protein entries) concatenated with reversed decoy sequences for false discovery rate

(FDR) control. Parameters follow the default setting of MS-GF+ except the following ones: 20 ppm for precursor mass tolerance, -1 to 2 for isotope error range, fully-tryptic peptides only, 2 to 7 for precursor charge state, cysteine carbamidomethylation for fixed modification, methionine oxidation and peptide N-terminal acetylation for variable modification. Peptide filtering, protein grouping and protein-level FDR control were conducted by IDPicker (v3.1.643.0). Minimal peptide number for identification was set to 2, and global protein-level FDR was set to 1%. Filtered PSM/peptide/protein lists were exported and combined into a spectrum report using an in-house R script.

For quantification, rawfiles were imported into SIEVETM (v2.2, Thermo Scientific) for global chromatographic alignment by ChromAlign and quantitative feature generation by a direct ion-current extraction (DICE) method. To ensure reliable quantification, only features with high quality (e.g. S/N ratio >10) were generated, and ion intensities in each sample run was calculated individually. A customized R package, IonStarStat, was used to integrate quantitative features with identification results, perform dataset-wide normalization, remove outlier peptides from quantification, and aggregate peptide intensities to protein level. Protein ratios and p-values (from paired t-test) between ascites/PK ascites and media control were calculated manually in Excel. Significantly changed proteins were determined by protein fold change >1.5 and p-value <0.05. Gene ontology (GO) analysis was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources v6.7 (http://david.abcc.ncifcrf.gov).

Cytotoxicity Assay

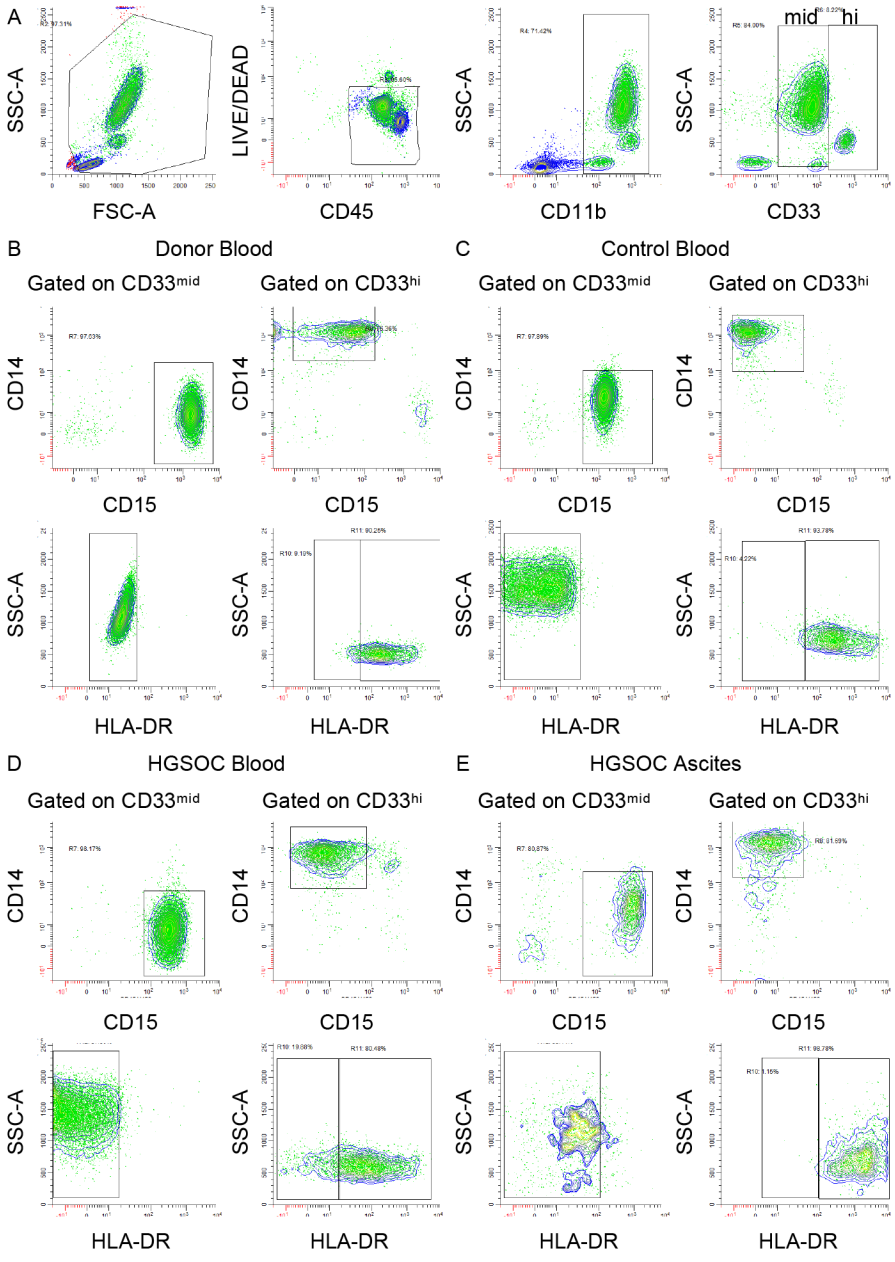
The *in vitro* cytotoxicity assay was performed using the CFSE-based assay, as previously described (43). Briefly, NY-ESO-1-specific $CD8^+$ T cells from patients who received NY-ESO-1 vaccination were amplified *in vitro* and NYESO-$1_{157-165}$-specific $CD8^+$ T cells were isolated. HLA-A*0201$^+$NYE-ESO-1$^-$ SK-MEL-29 (SK29) cells were pulsed with NY-ESO-$1_{157-165}$

peptide for 2h in an incubator at 37°C, 5% CO_2 followed by labeling with 0.5 µM CFSE. Peptide-unpulsed SK29 cells were labeled with 5 µM CFSE. Peptide-pulsed (2e4) and unpulsed (1:1) SK29 cells were cocultured with NY-ESO-$1_{157-165}$-specific $CD8^+$ T cells (4e4) in the presence or absence of ascites supernatants (50 µl) and/or PMN (1:1) for 16-18h. The cells were harvested by treatment with trypsin/EDTA, resuspended in buffer (DPBS, 1% heat-inactivated FBS), and stained with 7-AAD (BD Biosciences). The cells were acquired on a FACSCalibur flow cytometer (BD Biosciences) and the proportion of $CFSE^+7\text{-}AAD^-$ cells were analyzed by FlowJo software. Cytotoxicity was calculated using the following formula: % cytotoxicity = 100 x [1-(%$CFSE^{hi}$ peptide-unpulsed SK29/%$CFSE^{lo}$ peptide-pulsed SK29)$_{without\ T\ cells}$/(%$CFSE^{hi}$ peptide-unpulsed SK29/%$CFSE^{lo}$ peptide-pulsed SK29)$_{with\ T\ cells}$].

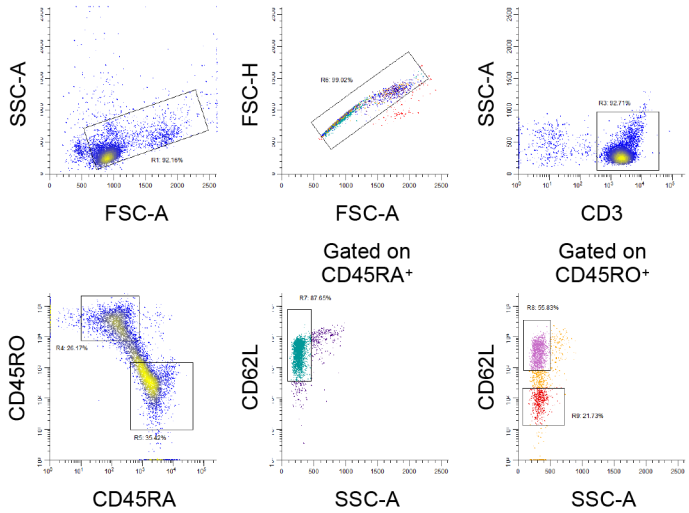
Engineered T Cells with NY-ESO-1-Specific TCR

Peripheral blood mononuclear cells (PBMC) from healthy donors were obtained through Ficoll separation. PBMC were activated with OKT3 (anti-CD3 Ab, 50 ng/mL) and rIL-2 (300 IU/mL) for 48h in an incubator at 37°C, 5% CO_2. Supernatants from the retrovirus construct MSCV-NY-ESO-1 TCR (collected from a stable PG13 producer cell line) were added to retronectin precoated plates, and the PBMC were introduced suspended in AIMV media (5% AB serum). The cells were centrifuged for spinoculation and kept for 16h in an incubator at 37°C, 5% CO_2, then washed and resuspended in fresh AIMV media (5% AB serum) for downstream applications.

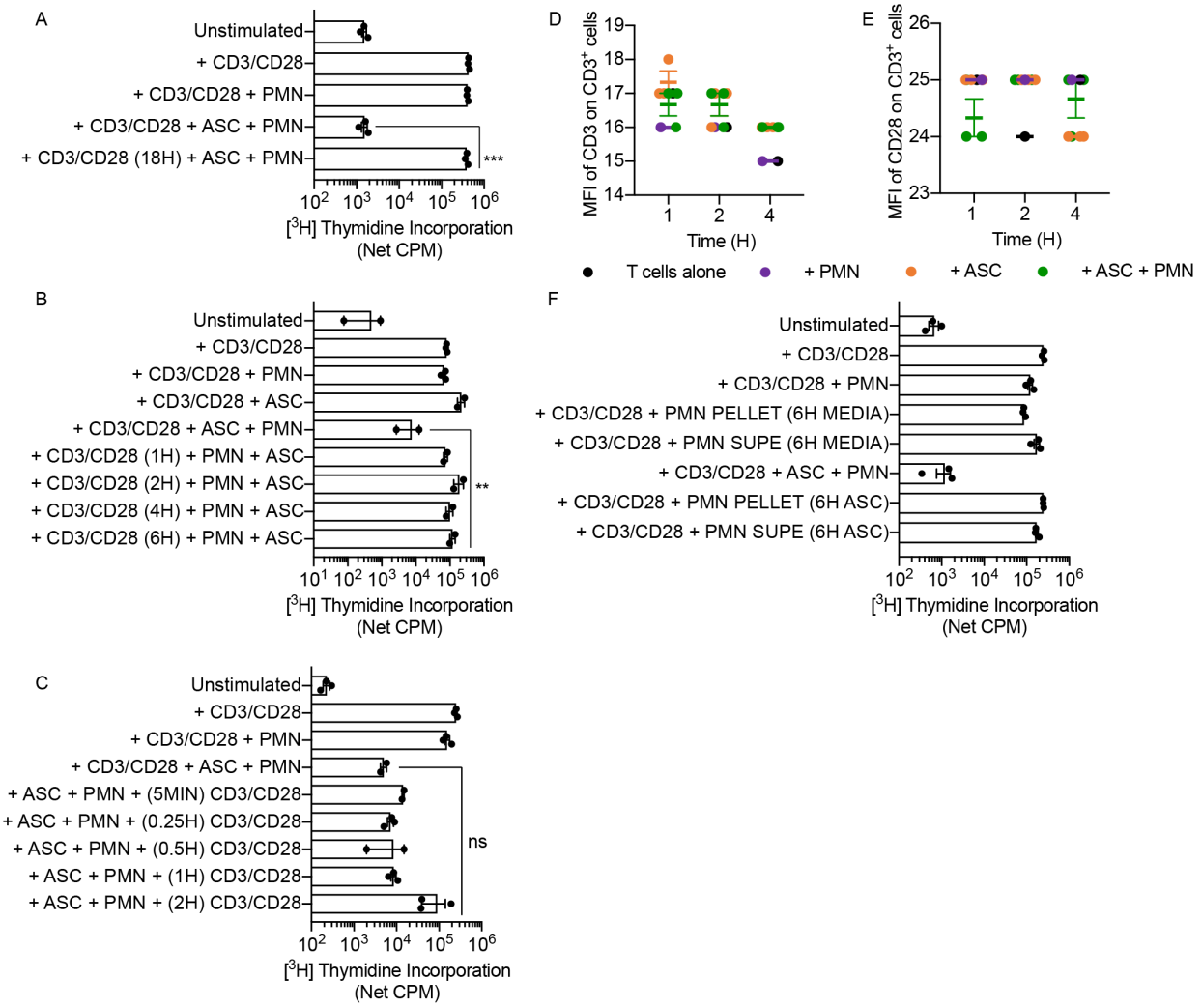
Figures and Legends



Supplemental Figure 1. Mature PMN in control blood and paired blood and ascites from patients with advanced HGSOC are CD33^{mid} (CD11b^{+}CD33^{mid}CD15^{+}CD14^{-}DR^{-}) and monocytes/macrophages are CD33^{high} (CD11b^{+}CD33^{hi}CD15^{-}CD14^{+}DR^{+}). A) Gating strategy. Representative raw data from B) donor blood, C) control blood, D) HGSOC blood, and E) HGSOC ascites.

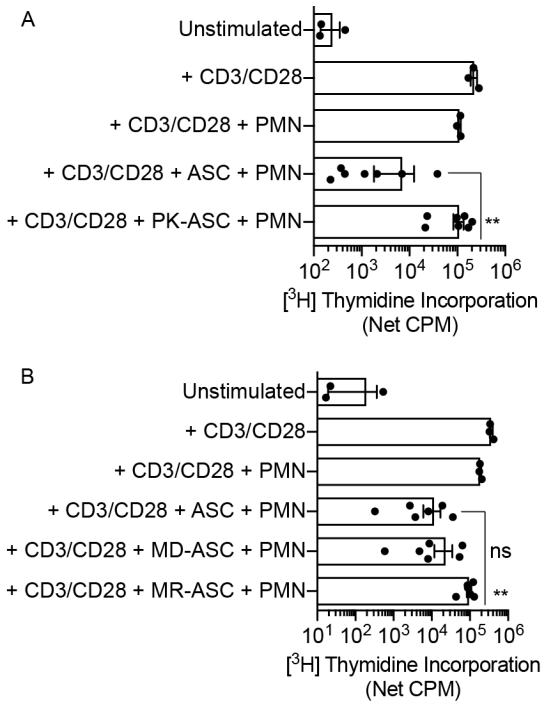


Supplemental Figure 2. Naïve, central memory, and effector memory T cells were flow sorted from healthy donor blood. Gating strategy for T cells: naïve ($CD3^+CD45RA^+RO^{neg}CD62L^+$), central memory ($CD3^+CD45RA^{neg}RO^+CD62L^+$), and effector memory ($CD3^+CD45RA^{neg}RO^+CD62L^{neg}$) populations.

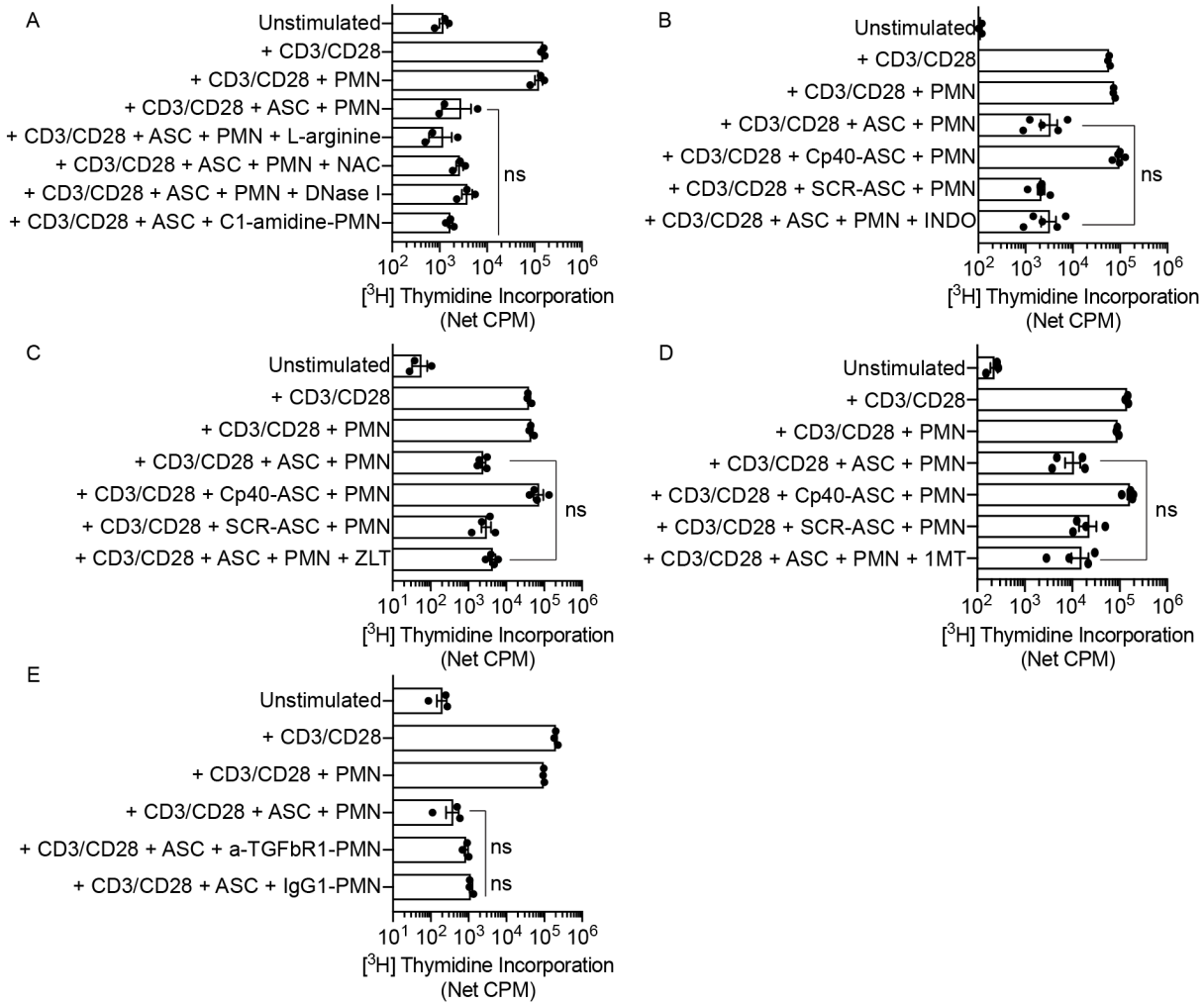


Supplemental Figure 3. PMN-mediated T cell suppression requires PMN and ascites at time of initial T cell stimulation. T cells ($CD3^+$) and PMN were used in autologous coculture at 1:1. PMN and/or ascites supernatants (ASC; 50% final well volume) were added to anti-CD3/CD28-stimulated T cells. After 72h of coculture, T cell proliferation was measured by [^{3}H] thymidine incorporation (16-18h). The coculture experiments described were modified. A) T cells stimulated with anti-CD3/CD28 beads for 18h prior to PMN and ASC addition are unable to be suppressed (n=3). B) T cells stimulated with anti-CD3/CD28 for 1-6h prior to PMN and ASC addition are unable to be suppressed (n=2). C) T cells were cocultured with ASC and PMN for 5 min to 2h prior to anti-CD3/CD28-stimulation (n=3). D-E) T cells were evaluated for surface expression of CD3 (D) and CD28 (E) after coculture with ASC and PMN for 1-4h (n=3). F) PMN were treated with ASC or media. After 6h, T cells and anti-CD3/CD28 beads were added to either pretreated PMN pellets or supernatants. Anti-CD3/CD28-stimulated T cell proliferation

was not suppressed by PMN pellets or supernatants, as compared to the t=0 coculture (n=2). Symbols represent individual samples (n) and bars represent SEM. Statistical comparisons were by ANOVA *(*, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant)*. Results were consistent between $CD4^+$ and $CD8^+$ T cells.

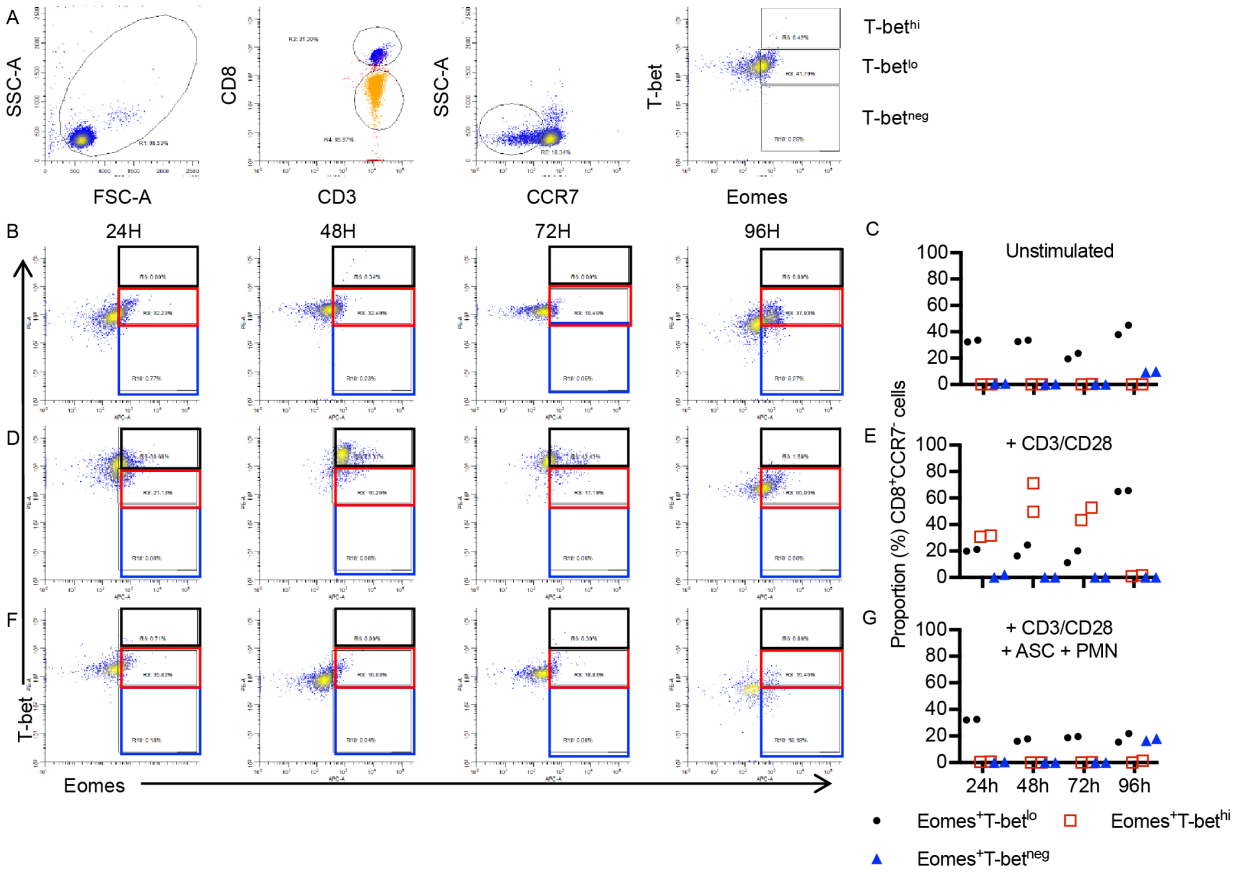


Supplemental Figure 4. Ascites supernatants contain soluble proteins that induce the PMN suppressor phenotype. T cells ($CD3^+$) and PMN were used in autologous coculture at 1:1. PMN and/or ascites supernatants (ASC; 50% final well volume) were added to anti-CD3/CD28-stimulated T cells. After 72h of coculture, T cell proliferation was measured by [^{3}H] thymidine incorporation (16-18h). Prior to coculture, ASC were treated as follows. A) Proteinase K-digested ASC (PK-ASC; 100 µg/ml) abrogated the PMN suppressor phenotype (n=12). B) ASC were ultra-centrifuged at 200,000g for 1.5h to fractionate membrane-associated proteins (200,000g pellets termed membrane-rich, MR-ASC; membrane-depleted supernatants termed MD-ASC). MD-ASC retained the ability to induce the PMN suppressor phenotype, as compared to unmanipulated ASC, and MR-ASC induced the PMN suppressor phenotype to a lesser extent, as compared to unmanipulated ASC (n=7). Symbols represent individual samples (n) and bars represent SEM. Statistical comparisons were by ANOVA *(*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ns, not significant).* Results were consistent between $CD4^+$ and $CD8^+$ T cells.

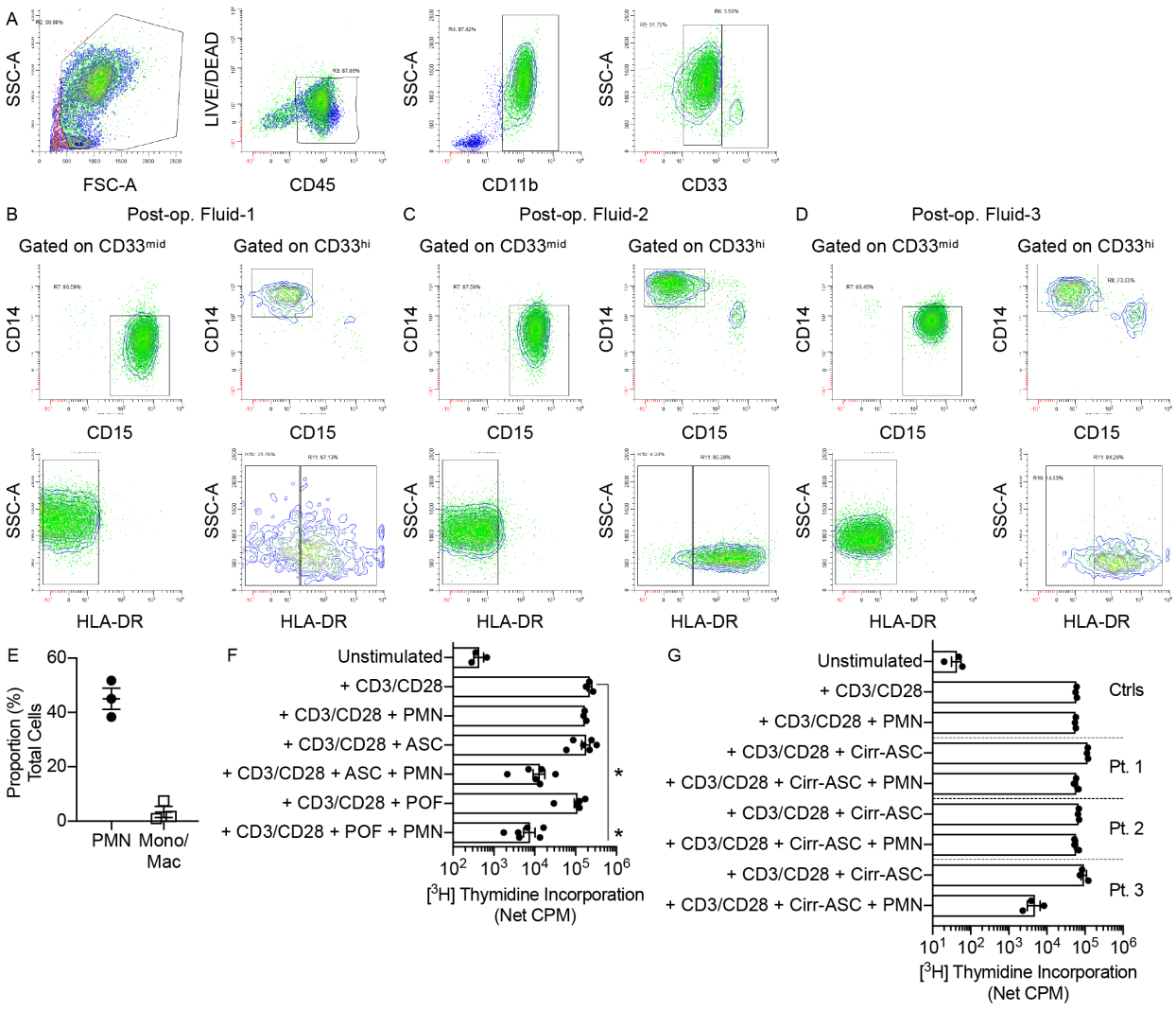


Supplemental Figure 5. Traditional pathways associated with granulocytic MDSC and N2 TAN do not play a role in the PMN suppressor phenotype. T cells ($CD3^+$) and PMN were used in autologous coculture at 1:1. PMN and/or ascites supernatants (ASC; 50% final well volume) were added to anti-CD3/CD28-stimulated T cells. After 72h of coculture, T cell proliferation was measured by [^{3}H] thymidine incorporation (16-18h). A) L-arginine (50 μM-1 mM), N-acetylcysteine (NAC; 10-25 mM), or DNase I (50-100 IU) added to cocultures did not reverse ascites-induced PMN-mediated suppression. PMN pretreatment with CI-amidine (10-20 μM) for 1h prior to coculture also did not abrogate the PMN suppressor phenotype (n=3). B-D) Arachidonic acid metabolism does not play a role in the PMN suppressor phenotype.

Indomethacin (INDO; 10 μM; n=5) (B), zileuton (ZLT; 50 μM; n=4) (C), or 1-methyl-DL-tryptophan (1-MT; 100 μM; n=4) (D) added to cocultures did not reverse the phenotype. E) PMN treated with anti-TGF-beta receptor 1 (TGFbR1) Ab for 1h prior to coculture had no effect on T cell proliferation. Treatment of PMN with IgG1 isotype (1-10 μg) similarly had no effect on proliferation (n=5). Symbols represent individual samples (n) and bars represent SEM. Statistical comparisons were by ANOVA *(*, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant).* Results were consistent between $CD4^+$ and $CD8^+$ T cells.



Supplemental Figure 6. PMN suppressor phenotype inhibits upregulation of transcriptional factors responsible for effector cell differentiation. T cells ($CD3^+$) and PMN were used in autologous coculture at 1:1. PMN and/or ascites supernatants (ASC; 50% final well volume) were added to anti-CD3/CD28-stimulated T cells. At 24, 48, 72, and 96h of coculture, intracellular markers for transcriptional control by T-bet and Eomes were evaluated on $CD8^+$ T cells. A) Gating strategy to delineate effector T cells ($CD8^+CCR7^-Eomes^+T\text{-}bet^{hi}$). B-G) Representative flow plots and quantification are shown for T cells that are B-C) unstimulated, D-E) stimulated, and F-G) stimulated with ASC and PMN (n=3). Symbols represent individual samples (n) and bars represent SEM.



Supplemental Figure 7. Inflammation and injury, whether resulting from the tumor microenvironment or other pathologic conditions, can induce the PMN suppressor phenotype. A-F) Post-operative drainage fluid was collected 1d after debulking surgery for EOC. A) Gating strategy. B-D) Representative raw data from each post-operative drainage fluid that had sufficient cells for analysis. E) Post-operative drainage fluid is composed primarily of PMN ($CD11b^{+}CD33^{mid}CD15^{+}CD14^{neg}DR^{neg}$) with minimal proportions of monocytes/macrophages ($CD11b^{+}CD33^{hi}CD15^{neg}CD14^{+}DR^{+}$) (n=3). F) Paired ASC or post-operative drainage supernatants (POF; 50% final well volume) were added to anti-CD3/CD28-stimulated donor T cells and/or autologous donor PMN. After 72h of co-culture, T cell

proliferation was measured by [^{3}H] thymidine incorporation (16-18h). ASC and POF equally induce the PMN suppressor phenotype (n=7). G) Ascites were collected from patients with cirrhosis and without cancer (n=3). 1/3 cirrhotic ascites supernatants (Cirr-ASC) induced the PMN suppressor phenotype. Statistical comparisons were by ANOVA.