Supplemental data

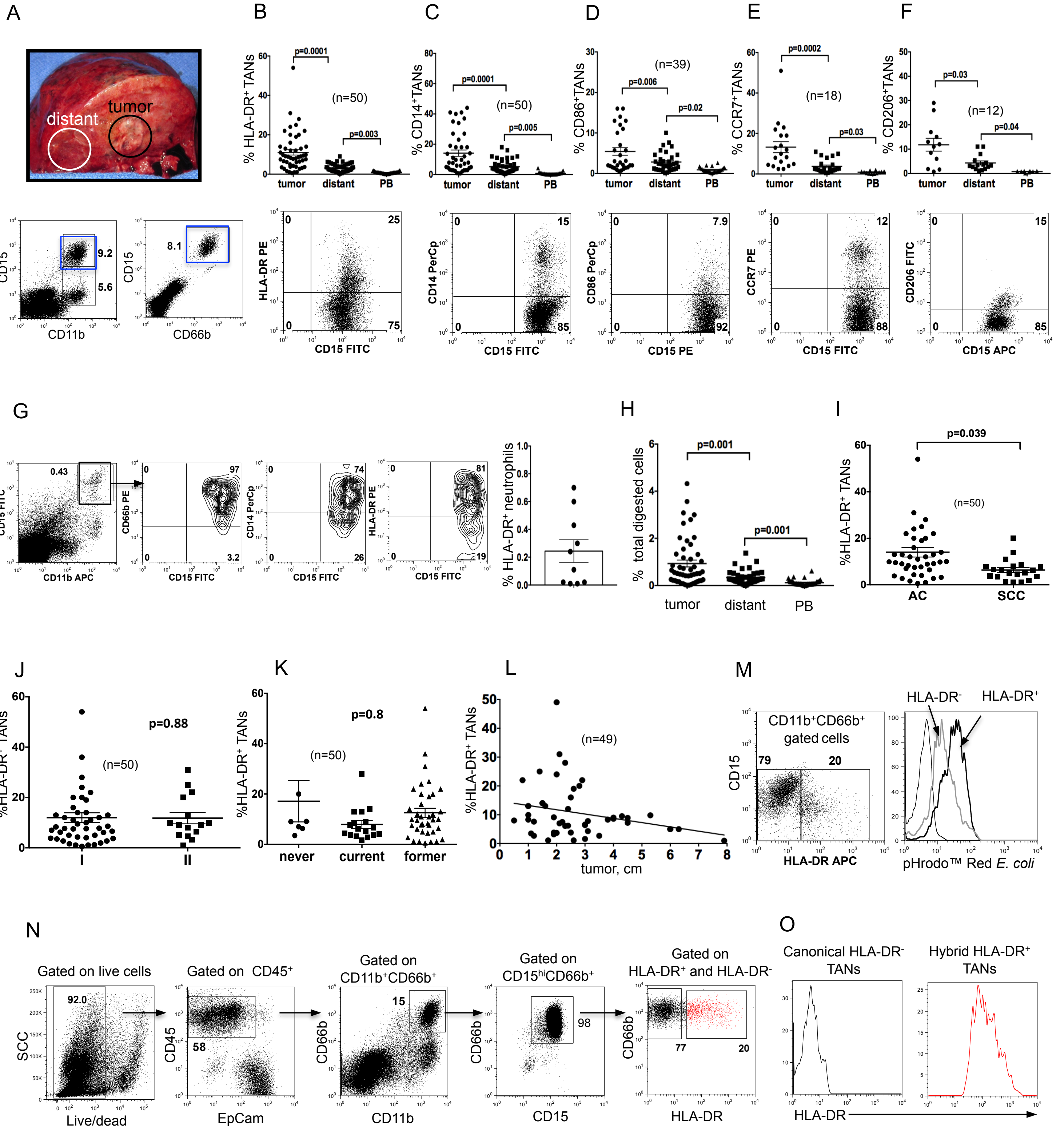
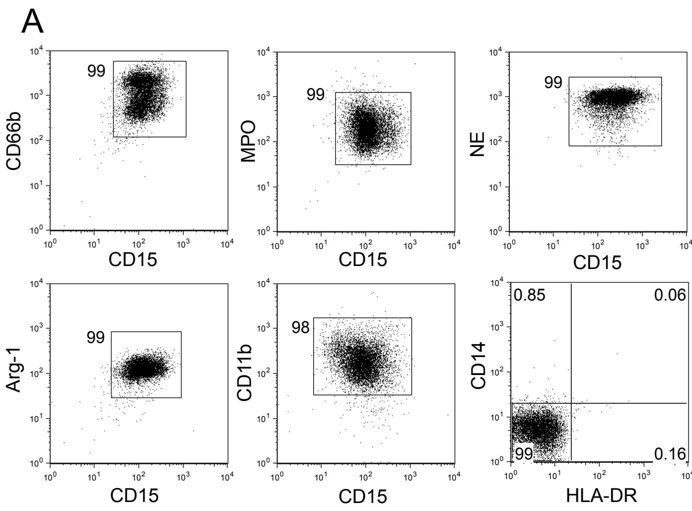


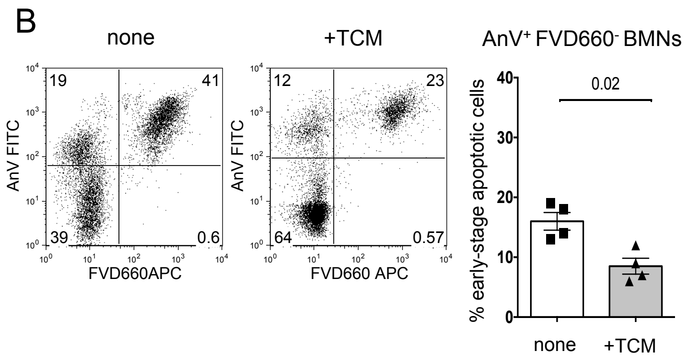
Figure S1 related to Figure 1. A subset of TANs with a hybrid characteristics of neutrophils and APCs.
(A) The photograph of the excised lung showing the location of tumor and distant adjacent tissues used for experiments. Dot plots represents the frequency of live $CD11b^{+}CD15^{hi}$ $CD66b^{+}$ TANs (blue boxes) in digested tumor tissue.
(B-F) The expression of HLA-DR (B), CD14 (C), CD86 (D), CCR7 (E), and CD206 (F) on gated $CD11b^{+}CD15^{hi}CD66b^{+}$ TANs (tumor), distant lung neutrophils (distant), and PBNs (PB). Top: Summary of all patient data. Data are presented as the percentage of cells among all TANs. Error bars represent mean ± SEM, 1-way ANOVA with Tukey's multiple comparison test. Bottom: Representative dot plots. TANs were defined in (A) as live $CD11b^{+}CD15^{hi}CD66b^{+}$ cells.
(G) The presence of APC-like hybrid neutrophils in the regional lymph nodes (LNs) of lung cancer patients. LNs were mashed through the cell strainer and single cell suspension was stained for indicated markers. Cells were gated on live $CD11b^{+}CD15^{hi}$ (black box) and further analyze for the expression of CD66b, CD14, and HLA-DR. Representative dot plots are shown. The error bars represent the mean ± SEM, n=10.
(H) The frequency of live APC-like hybrid TANs among all nucleated cells in (tumor), distant lung (distant) and peripheral blood (PB). Cumulative results from 50 independent experiments are shown in the scatter plots. The error bars represent the mean ± SEM. Statistical analyses were performed with repeated measures one-way ANOVA with Tukey's multiple comparison test.
(I-L) The frequency of APC-like hybrid TANs in patients with NSCLC with different tumor type (I) (AD-adenocarcinoma, SCC-squamous cell carcinoma), stage (J), smoking history (K) and size (L). The error bars represent the mean ± SEM, unpaired t test for (I) and (J), Kruskal-Wallis multiple comparison test for (K), nonparametric Spearman correlation for (L).
(M) Phagocytic activity of hybrid and canonical TANs. TANs were isolated from tumor and incubated with pHrodo™ Red *E. coli* BioParticles® for 45 min to allow phagocytosis (internalized particles become fluorescent [red]). The level of phagocytosis was measured in gated $HLA\text{-}DR^{-}$ canonical (grey line) and $HLA\text{-}DR^{+}$ hybrid TANs (black line). Representative results of 1 of 4 experiments are shown.
(N and O) Gating strategy for sorting of canonical $HLA\text{-}DR^{-}$ and hybrid $HLA\text{-}DR^{+}$ TANs by flow cytometry. A single cell suspension was obtained from freshly harvested tumor, stained for indicated markers and sorted based on the phenotype of canonical
($CD11b^{+}CD66b^{+}CD15^{hi}HLA\text{-}DR^{-}$) and hybrid ($CD11b^{+}CD66b^{+}CD15^{hi}HLA\text{-}DR^{+}$) TANs.

Cancer Type:	
Adenocarcinoma	73
Squamous Cell Carcinoma	36
Age:	
Median	67
Average	67.9
Range	52-88
Sex:	
Male	66
Female	43
Race:	
White	74
Black	26
Hispanic	1
Other	8
Tumor Stage:	
Stage IA	49
Stage IB	31
Stage IIA	20
Stage IIB	9
Tumor Grade:	
T1a	39
T1b	18
T2a	34
T2b	10
T3	8
Nodal Stage:	
N0	96
N1	13
N2	0
Smoking History:	
Current	29
Former	69
Never	10
Unknown	1

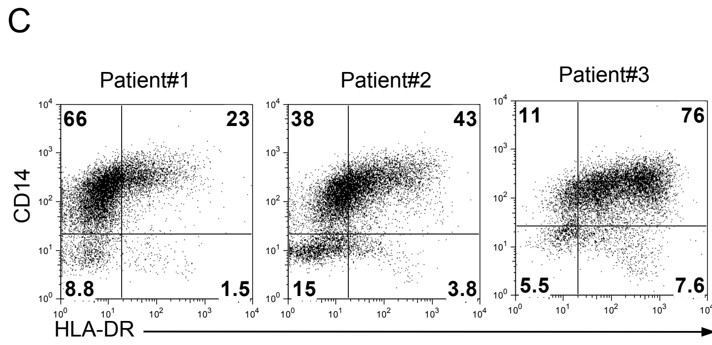
Table S1 related to Figure 1. Patient characteristics (n=109)



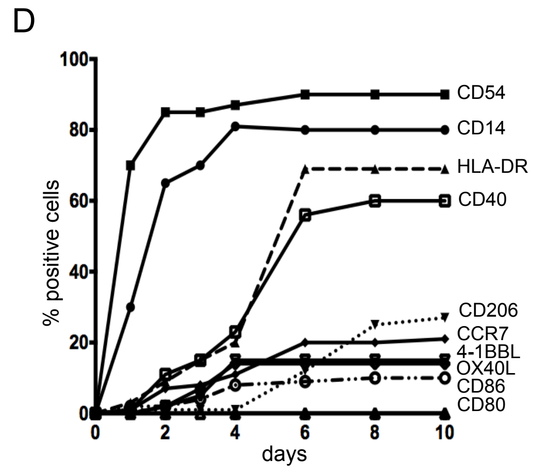
A
CD66b
CD15
99
MPO
NE
Arg-1
CD11b
98
CD14
HLA-DR
0.85
0.06
0.16



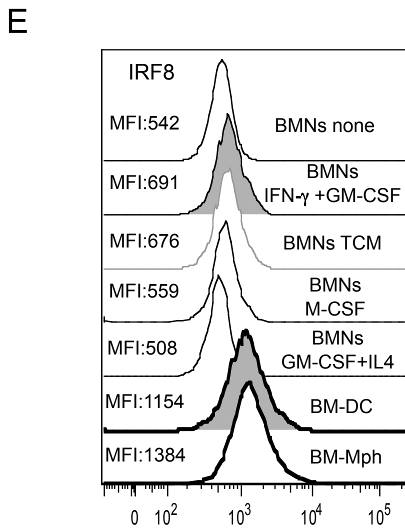
B
none
+TCM
AnV+ FVD660- BMNs
AnV FITC
FVD660APC
FVD660 APC
19
41
39
0.6
12
23
64
0.57
% early-stage apoptotic cells
0.02
none
+TCM



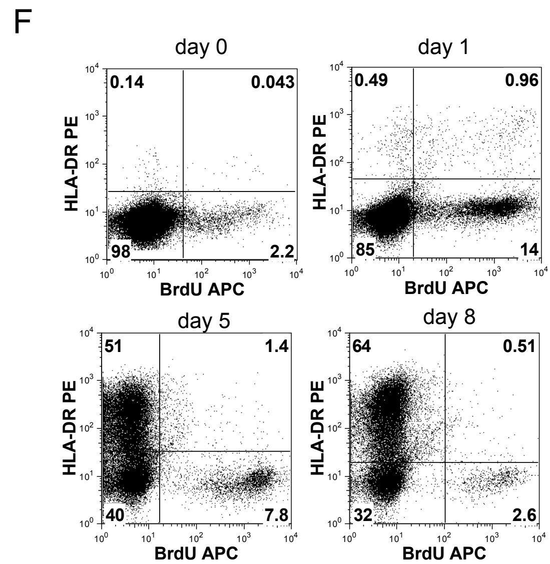
C
Patient#1
Patient#2
Patient#3
CD14
HLA-DR
66
23
8.8
1.5
38
43
15
3.8
11
76
5.5
7.6



D
% positive cells
days
CD54
CD14
HLA-DR
CD40
CD206
CCR7
4-1BBL
OX40L
CD86
CD80



E
IRF8
MFI:542
BMNs none
MFI:691
BMNs IFN-γ +GM-CSF
MFI:676
BMNs TCM
MFI:559
BMNs M-CSF
MFI:508
BMNs GM-CSF+IL4
MFI:1154
BM-DC
MFI:1384
BM-Mph



F
day 0
day 1
day 5
day 8
HLA-DR PE
BrdU APC
0.14
0.043
98
2.2
0.49
0.96
85
14
51
1.4
40
7.8
64
0.51
32
2.6

Figure S2 related to Figure 2. Tumor-derived factors differentiate long-lived immature BMNs into a hybrid subset with a partial phenotype of dendritic cells and macrophages

(A) Flow cytometric analysis of the expression of CD15, CD66b, CD11b, intracellular MPO, NE and Arg1 on freshly isolated BMNs. Representative dot plots of 1 of 8 experiments are shown.

(B) BMN survival in vitro. BMNs were incubated with or without hybrid-inducing TCM. Seven days later, BMNs were stained with viability dye FVD 660 followed by staining for AnnexinV and analyzed by flow cytometry. The error bars represent the mean ± SEM, Wilcoxon matched-pairs rank test, n=4.

(C) BMNs were isolated from three different cancer patients and treated with the same hybrid-inducing TCM collected from patient#78. The expression of HLA-DR^{+} and CD14^{+} was measured on gated live CD11b^{+}CD66b^{+}CD15^{hi} BMNs by flow cytometry.

(D) Kinetic of indicated APC marker expression in BMNs treated with hybrid-inducing TCM. The expressions of indicated markers were assessed by flow cytometry on live CD11b^{+}CD66b^{+}CD15^{hi} BMNs for different time points. Results represent 1 of 7 similar experiments.

(E) Comparative analysis of IRF8 expression (presented as Mean Fluorescence Intensity (MFI) histograms) in non-treated BMNs (BMNs none) and BM-derived hybrid neutrophils differentiated with IFN-γ and GM-CSF (BMNs IFN-γ+GM-CSF) or TCM (BMNs TCM). BMNs neutrophils treated with M-CSF and GMCSF/IL-4 were used as negative control whereas BM-derived macrophages (Mph) and dendritic cells (DC) were used as positive control. Representative dot plots from 1 of 6 experiments are shown.

(F) Proliferation of HLA-DR^{-} and HLA-DR^{+} BMNs in vitro in the presence of hybrid–inducing TCM. BMNs were exposed to hybrid-inducing TCM for 8 days. One, five and eight days later, the proliferation of neutrophils was assessed by intracellular staining of incorporated BrdU into DNA of HLA-DR^{-} and HLA-DR^{+} BMNs. The expression of HLA-DR was measured on gated live CD11b^{+}CD66b^{+}CD15^{hi} BMNs. Representative results from 1 of 3 experiments are shown.

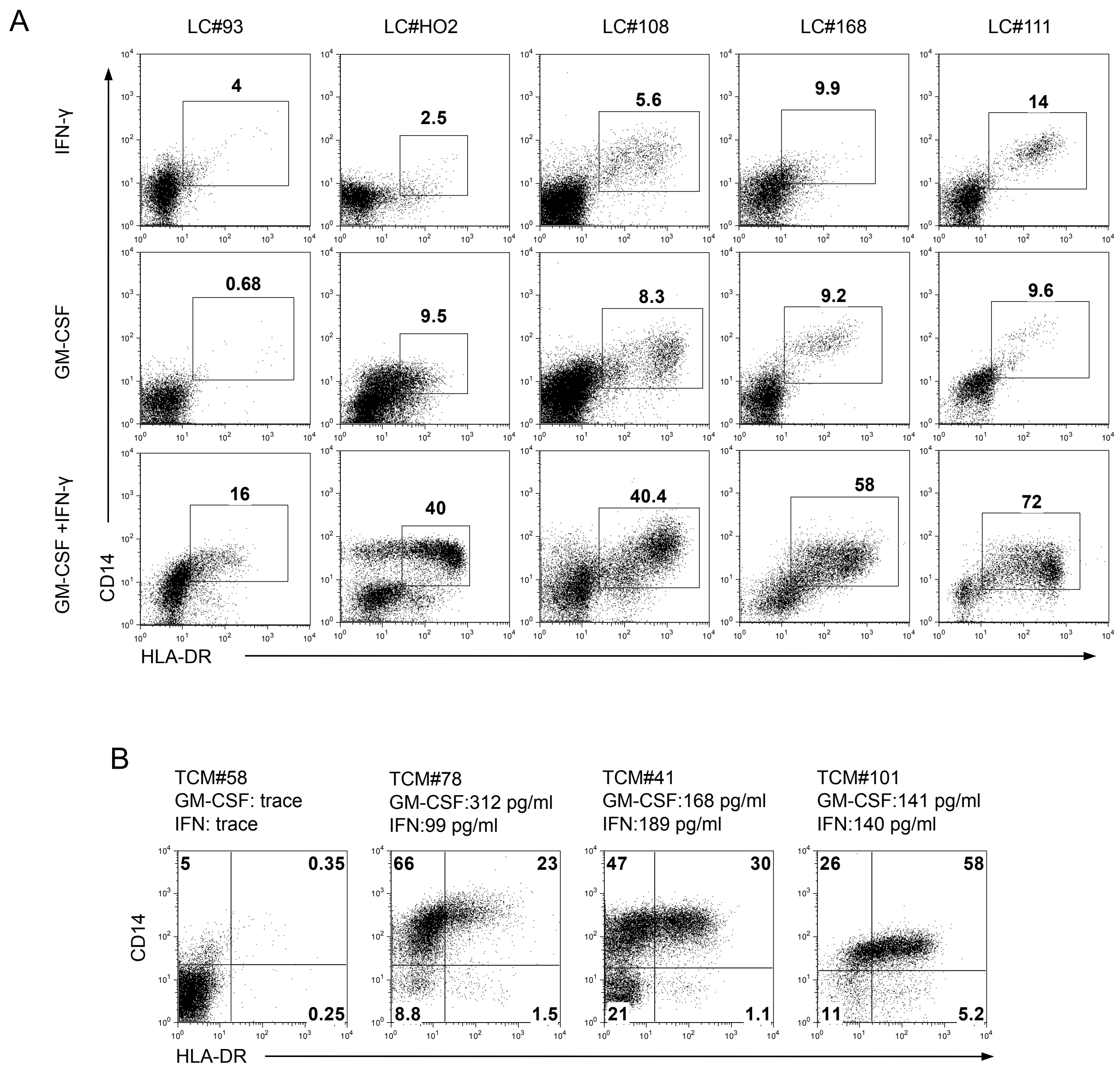
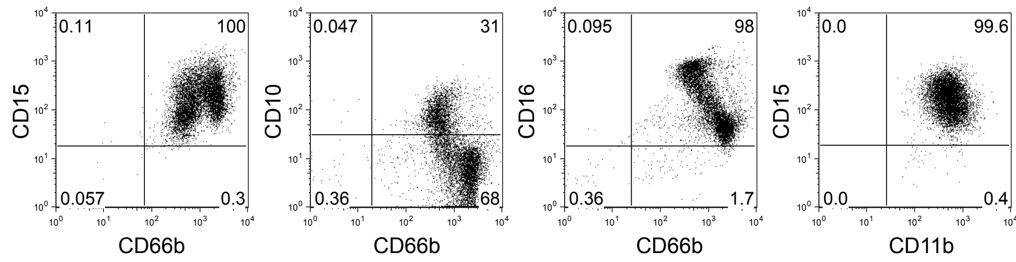


Figure S3 related to Figure 3. Tumor-derived IFN-γ and GM-SCF synergistically differentiate immature neutrophils into a subset of APC-like hybrid neutrophils.

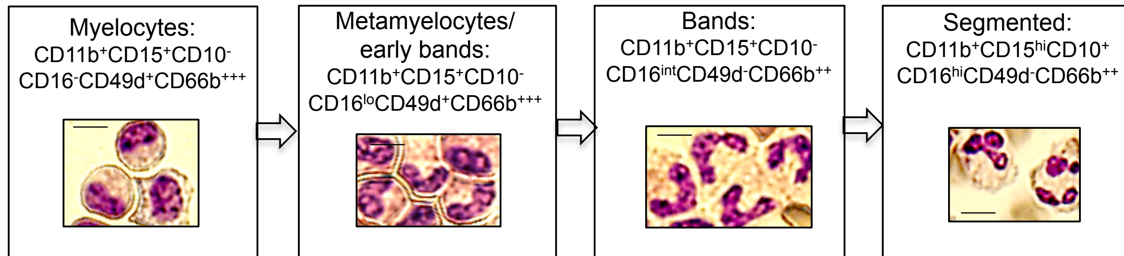
(A) Flow cytometric analysis of the expression of CD14 and HLA-DR markers on gated live $CD11b^{+}CD66b^{+}CD15^{hi}$ BMNs differentiated in the presence of IFN-γ (50 pg/ml) and GM-CSF (50 pg/ml) for 5 days. BMNs isolated from five different lung cancer patients are shown.

(B) The effect of TCMs with different concentration of GM-CSF and IFN-γ on the formation of hybrid $HLA\text{-}DR^{+}CD14^{+}$ BMNs. BMNs were isolated from one cancer patient and treated with hybrid-inducing TCM collected from different patients (#58, #78, #41, #101). The expression of HLA-DR and CD14 was measured on gated live $CD11b^{+}CD66b^{+}CD15^{hi}$ BMNs. Concentration of GM-CSF and IFN-γ in TCMs was quantified by ELISA.

A



B



C

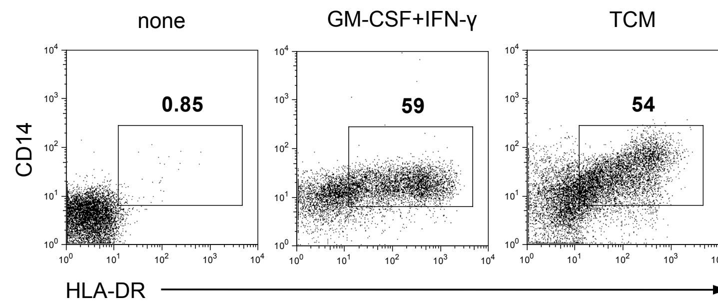


Figure S4 related to Figure 4. APC-like hybrid neutrophils originate from CD11b^{+}CD15^{hi}CD66b^{+}CD10^{-}CD16$^{lo/int}$ progenitors

(A) The co-expression of CD15, CD66b, CD11b, CD16 and CD10 was analyzed by flow cytometry on freshly isolated BMNs. Representative dot plots from 1 of 5 experiments are shown.

(B) Schematic representation of the phenotype and nuclear morphology of CD11b^{+}CD15^{hi} BMNs at different stages of development. BMNs at different stages of maturation were isolated by flow cytometry sorting and analyzed for the indicated surface markers by flow cytometry. Cytospins were made from sorted BMNs and stained with the Hema3 Stat Pack Kit (Wright-Giemsa-like stain).

(C) The formation of hybrid HLA-DR^{+}CD14^{+} neutrophils from G-CSF mobilized low density immature PBNs. The expression of HLA-DR and CD14 was measured on gated live CD11b^{+}CD66b^{+}CD15^{hi} PBNs after the treatment with hybrid-inducing TCM or IFN-γ (50 pg/ml) and GM-CSF (50 pg/ml). Representative dot plots from 1 of 4 similar experiments are shown.

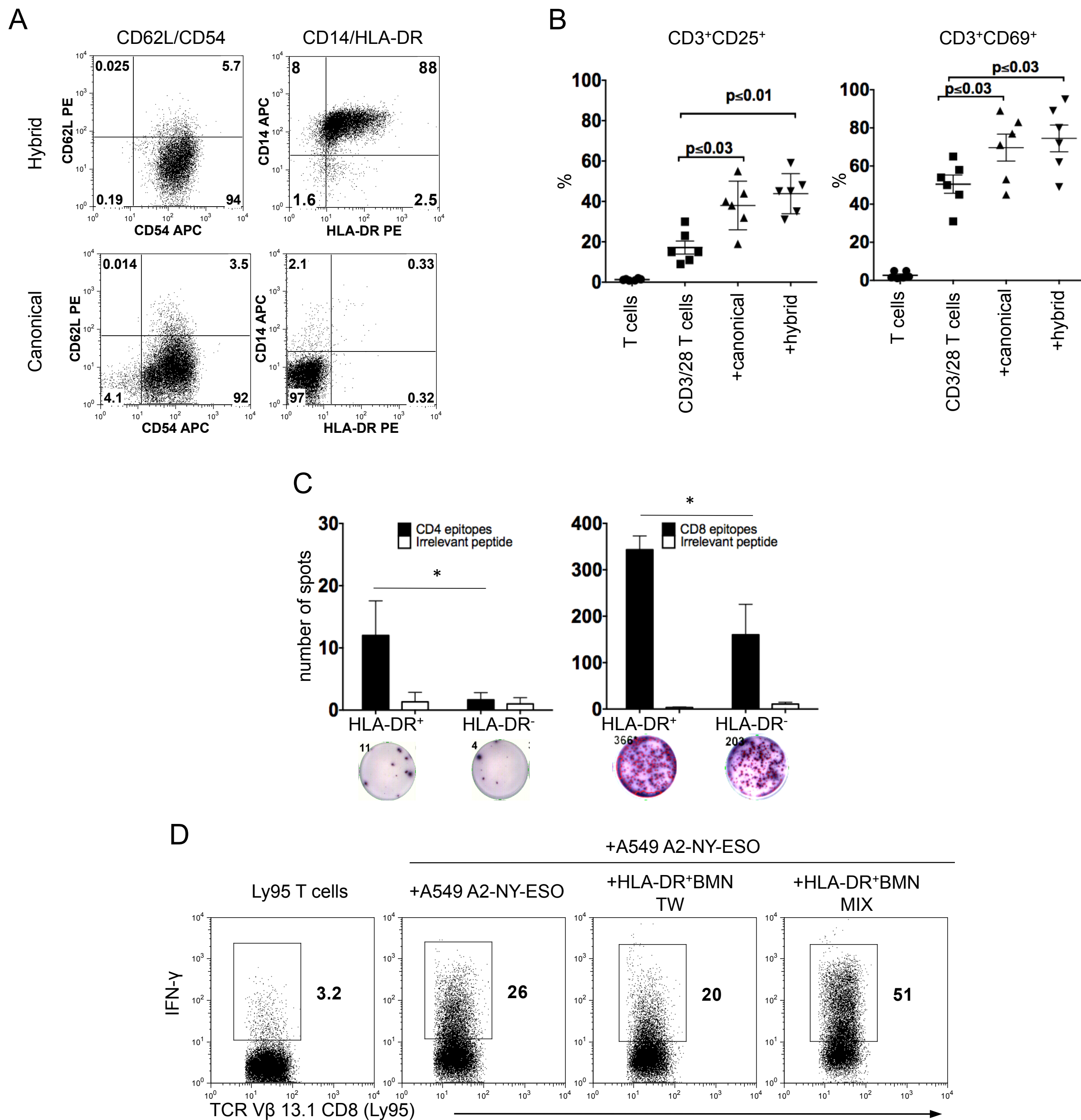


Figure S5 related to Figure 6. APC-like hybrid neutrophils are able to stimulate T cell responses.

(A) Development of hybrid and canonical neutrophils. To obtain hybrid HLA-DR^{+}CD14^{+} neutrophils (top panel), BMNs were treated with hybrid-inducing TCM collected from tumor digests where the frequency of hybrid TANs was markedly elevated. To obtain canonical HLA-DR^{-}CD14^{-} neutrophils (bottom panel), BMNs were treated with TCM collected from tumor digests where hybrid TANs were not detected. The expression of CD62L, CD54, HLA-DR and CD14 was measured by flow cytometry on gated live CD11b^{+}CD66b^{+}CD15^{hi} cells. Representative dot plots from 1 of 12 experiments are shown.

(B) Expression of the CD25, and CD69 markers on activated autologous T cells co-incubated with BM-derived canonical and hybrid neutrophils. T cells were isolated from PBMC, stimulated with plate-bound anti–CD3 Abs and incubated with BM-derived neutrophils at a 1:1 ratio for 24 hours. Error bars represent mean ± SEM from 6 independent experiments (Wilcoxon matched-pairs rank test).

(C) Ability of hybrid HLA-DR^{+} or canonical HLA-DR^{-} BMNs to stimulate autologous virus-specific memory T cell response in an IFN-γ–ELISPOT assay. Autologous T cells were isolated from PBMC and co-cultured with BM-derived canonical HLA-DR^{-} and hybrid HLA-DR^{+} neutrophils that had been pulsed with a mixture of viral T cell epitopes for 2 hours. The number of IFN-γ–producing T cells was determined in three independent ELISpot assays. Error bars represent mean ± SEM from 3 independent experiments (*$p \leq 0.01$, Mann-Whitney test).

(D) Flow cytometric analysis of IFN-γ production by Ly95 T cells stimulated with A2/NY-ESO A549 tumor cells in the presence of hybrid HLA-DR^{+} BMNs using a transwell system. Activated Ly95 T cells were mixed with HLA-DR^{+} BMNs at a 1:1 ratio (mix). To separate T cells and BMNs, activated T cells were cultured in the bottom chamber and HLA-DR^{+} BMNs were placed in the top chamber of the 24-well flat-bottom transwell culture plate (TW). Representative results from 1 of 3 experiments are shown.

Supplemental Experimental Procedures

Study Design

A total of 109 patients with Stage I-II lung cancer, who were scheduled for surgical resection, were consented for tissue collection of a portion of their tumor and/or blood for research purposes at the Hospital of the University of Pennsylvania and The Philadelphia Veterans Affairs Medical Center after obtaining consents that had been approved by their respective Institutional Review Boards. All patients selected for entry into the study met the following criteria: (i) histologically confirmed pulmonary squamous cell carcinoma (SCC) or adenocarcinoma (AC), (ii) no prior chemotherapy or radiation therapy within two years, and (iii) no other active malignancy. Detailed characteristics of the patients can be found in Table S1.

Reagents

The enzymatic cocktail for tumor digestion consisted of serum-free Hyclone ™ Leibovitz L-15 media supplemented with 1% Penicillin-Streptomycin, Collagenase type I and IV (170 mg/L=45-60 U/mL), Collagenase type II (56 mg/L=15-20 U/mL), DNase-I (25 mg/L), and Elastase (25 mg/L) (all from Worthington Biochemical, NJ). Complete cell culture media DME/F-12 1:1 media (HyClone, Thermo Scientific) was supplemented with 2.5 mM L-glutamine, 15 mM HEPES Buffer, 10% of Embryonic Stem (ES) Cell Screened FBS (U.S.) (Thermo Scientific™ HyClone™), Penicillin (100 U/ml) and Streptomycin (100 μg/mL). HLA-A*0201-restricted NY-ESO-1 (157-165, SLLMWITQV) peptide was synthesized by AnaSpec, Inc (Fremont, CA). Pierce™ NY-ESO-1 full-length recombinant protein and anti-NY-ESO-1 monoclonal Abs (clone E978 IgG1) were purchased from Thermo Scientific™. The PepMixCEF-MHC class I peptide pool (23 viral peptides) and The PepMixCEFT-MHC class II peptide pool (14 viral peptides) were purchased from JPT Peptide Technologies (Acton, MA). These peptide pools contain MHC class I and class II-restricted T-cell epitopes from CMV, EBV and Influenza virus, designed to stimulate T cells from donors with a variety of HLA types. Human recombinant IFN-γ, GM-CSF, IL-4 and M-CSF were purchased from PeproTech, Inc.

Preparation of a single-cell suspension from tumor and adjacent lung tissue

Surgically-removed fresh lung tumors and adjacent lung tissue were processed within 20 minutes of removal from the patient. We used our new optimized disaggregation method for human lung tumors that preserves the phenotype and function of the immune cells as previously described in details (Quatromoni, et al, 2015). Briefly, under sterile conditions, all areas of tissue necrosis were trimmed away. The tumor and adjacent uninvolved lung tissue was sliced into 1–2 mm^3 pieces with micro-dissecting scissors equipped with tungsten carbide insert blades (Biomedical Research Instruments, Inc. Silver Spring, MD). For enzymatic digestion, the pieces were incubated in a shaker for 45 minutes at 37°C in serum-free L-15 Leibovitz media (HyClone) containing different enzymes at low concentrations (see specifics above) and 1% Penicillin-Streptomycin (Life Technologies, Carlsbad, CA). L-15 Leibovitz media was formulated for use in carbon dioxide-free systems. After 45 minutes, any visible tumor pieces were vigorously pipetted against the side of a 50 mL tube to enhance disaggregation and then further incubated for 30-50 minutes under the same conditions. Larger pieces of tumor tissue were permitted to settle to the bottom of the tube and the supernatant was passed through a 70 μM nylon cell strainer (BD Falcon). The remaining pieces in the tube underwent further pipetting before being passed through the same cell strainer. Typically, less than 5% of the tissue (consisting of chiefly non-cellular connective tissue) remained on the cell strainer. After filtration the red blood cells were lysed using 1x Red Blood Cell (RBC) Lysis Buffer (Santa Cruz, Dallas, TX). The remaining cells were washed twice in RPMI supplemented with 2% FBS and re-suspended in the cell culture media. Cell viability, as determined by trypan blue exclusion or Fixable Viability Dye eFluor® 450 staining, was typically >90%. If the viability of cells was less than 80%, dead cells were eliminated using a "dead cell removal kit" (Miltenyi Biotec Inc., Germany).

Tumor-Conditioned Media

A single-cell suspension was obtained from lung tumors by enzymatic digestion as described above. After washing the cells with PBS, the single cell suspensions were re-suspended in DMEM/F12 (HyClone) medium supplemented with 5% FBS/antibiotics (penicillin/ streptomycin, HyClone) and placed

in 175 mm^2 flasks at a concentration of 2 x 10^6 cells/mL. Twenty-four hours later, supernatant (tumor-conditioned medium, TCM) was collected, filtered, aliquoted, and frozen at -80°C.

Neutrophil isolation

Since temperature gradients can activate neutrophils, all tissues and reagents were maintained at a constant temperature during preparation. After tumor harvest, the neutrophil populations used in this study were prepared at room temperature (RT) and rapidly utilized.

TANs were isolated from tumor single-cell suspensions using positive selection of $CD15^+$ or $CD66b^+$ cells with microbeads as previously described (Eruslanov, et al, 2014). In the rare instances when cellular aggregates formed, the suspensions were passed through a 30 μM pre-separation filter (Miltenyi) before addition to the LS columns (Miltenyi). For positive selection of TANs through engagement of the CD15 transmembrane protein, single cell suspensions were incubated with anti-CD15 antibody (Ab)-conjugated magnetic microbeads (Miltenyi Biotec) for 15 minutes. For positive selection of TANs through engagement of the CD66b transmembrane protein, single cell suspensions were first incubated with PE-conjugated anti-CD66b Abs (Biolegend) and then with anti-PE microbeads (Miltenyi Biotec). In some experiments, TANs were isolated by flow cytometric cell-sorting based on the phenotype of TANs as $CD11b^+CD66b^+CD15^{hi}$. Neutrophils from distant non-involved lung tissue were isolated similarly to TANs.

TAN subsets were sorted based on the phenotype of canonical ($CD11b^+CD66b^+CD15^{hi}HLA\text{-}DR^-$) and hybrid ($CD11b^+CD66b^+CD15^{hi}HLA\text{-}DR^+$) TANs. Gating strategy for flow cytometry sorting of canonical and hybrid TANs is shown in Figure S1N and S1O. $CD11b^+$ myeloid cells are all $CD45^+EpCam^-$ cells. Sterile cell sorting was performed on the BD FACSAria II (BD Biosciences) and MoFlo® Astrios™ (Beckman Coulter).

PBNs were obtained from EDTA anti-coagulated peripheral blood collected from lung cancer patients during surgery or from healthy donors. The PBNs were obtained from Lymphoprep (Accu-Prep, 1.077 g/ml, Oslo, Norway) density gradient centrifugation followed by erythrocyte lysis with 1x RBC Lysis Buffer. To account for any possible effect of tissue digestion enzymes on the function neutrophils, peripheral blood granulocytes were processed in a similar manner. Specifically, peripheral blood granulocytes were incubated with enzymatic cocktail before positive selection using microbeads or flow cytometry.

BMNs were isolated from bone marrow cell suspensions using positive selection of $CD15^+$ or $CD66b^+$ cells with microbeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA)**.** Bone marrow cell suspension was obtained from the rib fragments that were removed from patients as part of their lung cancer surgery. The single cell suspension was obtained by vigorous pippeting of cells flushed from bone marrow and passing the disaggregated cells through a 70 μM nylon cell strainer. To exclude the possible contamination of common progenitors, neutrophils were isolated from a CD34-depleted population of bone marrow cells. Anti-CD15 Ab-conjugated magnetic microbeads (Miltenyi Biotec) or PE-conjugated anti-CD66b Abs (Biolegend) and anti-PE microbeads (Miltenyi Biotec) were used for positive selection. Given that resting naïve neutrophils do not tightly adhere to cell culture plastic as opposed to macrophage and monocytes we additionally cultured the bead sorted BM neutrophils in cell culture dishes to exclude the possible contamination of BM macrophages/monocytes. Two-four hours later, the floating cells were removed and used for further experiments.

The purity and activation status of isolated TANs, BMNs and PBNs were measured by flow cytometry for the granulocyte/myeloid markers CD66b, CD15, arginase-1 (Arg), myeloperoxidase (MPO), CD11b, and the activation markers CD62L and CD54 as described earlier (Eruslanov, et al, 2014). All neutrophil subsets demonstrated high cell viability with minimal enzyme-induced premature cellular activation or cleavage of myeloid cell markers. The purity of TANs, BMNs and PBNs was typically higher than 94%. Isolates with less than 90% purity were discarded. To evaluate the cytomorphology of isolated PBNs, BMNs, and TAN subsets cells were spun on glass slides and stained with the Hema3 Stat Pack Kit (Fisher Scientific).

Lymphocyte isolation from Peripheral Blood

Standard approaches were utilized. Peripheral blood mononuclear cells (PBMCs) were separated by 1.077 g/ml Lymphoprep (Accu-Prep, Norway) gradient density centrifugation of EDTA anti-coagulated whole blood collected from cancer patients and healthy donors. T cells were purified from the PBMC

fraction using human T cell enrichment columns (R&D Systems, Inc.) according to the manufacturer's protocol.

Generation of BM-derived hybrid and canonical neutrophils

To differentiate BMNs into a cells that resemble canonical TANs, we culture the purified long-lived BMNs for 7 days with a TCM (50% v/v) collected from a patient's tumor digest where we were previously unable to identify a large number of hybrid TANs by flow cytometry. To differentiate BMNs into a cells that resemble hybrid TANs we culture the purified long-lived BMNs for 5-7 days with a TCM (50% v/v) collected from a tumor digest where the frequency of hybrid TANs was markedly elevated (≥ 15% of all TANs). Alternatively, hybrid-neutrophils were differentiated from BMNs with low doses IFN-γ (50 pg/ml) and GM-CSF (50 pg/ml) for 7 days. If we observed that the formation of hybrid BMNs was less that 80% we enriched the HLA-DR$^+$CD14$^+$ hybrid cells from TCM treated BMNs by positive selection using magnetic beads coated with anti-HLA-DR antibodies or by flow cytometric cell sorting. The proliferation of BMNs during the differentiation was assessed by flow cytometry using BrdU Flow Kit (BD Pharmingen). BMNs were exposed to bromo-deoxyuridine for 6 hours.

To test the effect of hypoxia on hybrid neutrophil formation, BMNs were cultured for 6 days under normoxic and hypoxic culture conditions maintained in a 37°C incubator containing 5% CO2, and either atmospheric 21% O2 or 5% O2 condition (Hypoxia Incubator Chamber, Stemcell Techology). We also cultured BMNs in the presence of hybrid-inducing TCM and cobalt chloride (25 μM) (MP Biomedicals LLC), an agent that induces the hypoxia-inducible factor-1α, the main transcriptional factor activated in hypoxic conditions.

To differentiate hybrid neutrophils from circulating immature neutrophils we used peripheral blood collected from healthy donors who were treated with G-CSF (filgrastim) in order to mobilize of hematopoietic stem cells for allogeneic hematopoietic cell transplantation. Peripheral blood mononuclear cells (PBMCs) were separated by 1.077 g/ml Lymphoprep (Accu-Prep, Norway) gradient density centrifugation of EDTA anti-coagulated whole blood collected from G-CSF treated healthy donors. Low-density immature neutrophils were isolated from PBMC using anti-CD15 microbeads and cultured with hybrid-inducing TCM for 7 days.

Generation of BM-derived macrophages and dendritic cells

Macrophages and dendritic cells were differentiated from myeloid CD11b$^+$cells purified with CD11b beads from CD15-depleted bone marrow cell suspensions. To obtain BM-derived mature dendritic cells (DC), CD11b cells were cultured in the presence of GM-CSF (25 ng/ml) and IL-4 (25 ng/ml) for 7 days in the complete cell culture medium, as described in detail elsewhere (Inaba, et al, 1992; Lutz, et al, 1999). Maturation cocktail (LPS 100 ng/ml and sOX40L 50 ng/ml) was added during the last 24 hours of cell culturing. To obtain BM-derived macrophages (Mph), BM CD11b cells were cultured in the presence of M-CSF in the complete cell culture medium for 7 days as described in detail elsewhere (Manzanero, 2012).

Flow Cytometry

Flow cytometric analysis was performed according to standard protocols. Matched isotype antibodies were used as controls. Negative gating was based on a fluorescence minus one (FMO) strategy. To exclude dead cells from analysis, cells were stained with the Fixable Viability Dye eFluor® 450 (e-bioscience), LIVE/DEAD® fixable dead cell stains (Molecular probes, Life Technologies), or Zombie Yellow™ Fixable Viability dye (Biolegend). To distinguish early-stage apoptotic and late-stage apoptotic/necrotic cells, cells were first stained with Fixable Viability Dye eFluor® 660 (eBioscience). Than cells were washed with the AnnexinV-Binding Buffer and stained with anti-AnnexinV Abs (FITC) in the AnnexinV-Binding Buffer for 10 min at RT. Cells were washed and analyzed by flow cytometry.

For intracellular staining, fixed cells stained for surface markers were permeabilized with BD Perm/Wash™ Buffer (BD Biosciences) and then stained with the following Abs for 45 minutes at RT: anti-human Arg (R&D Systems), anti-human MPO (e-bioscience), FITC-anti-human IFN-γ (Biolegend, clone: 4S.B3), APC anti-human GranzymeB (Biolegend, clone GB11) or PE-anti-human/mouse IRF8 (e-bioscience, Clone: V3GYWCH). For NE staining, fixed cells stained for surface markers were permeabilized with BD Perm/Wash™ Buffer (BD Biosciences) and then incubated with 1xPBS/10% normal goat serum (Abcam)/0.3M glycine to block non-specific protein interactions followed by the anti-human Neutrophil Elastase antibodies (EPR7479, Abcam) for 30 min at RT. The secondary Abs used were

goat anti-rabbit (Abcam) at 1/2000 dilution for 30 min at RT. Isotype control Abs were rabbit IgG used under the same conditions.

For transcription factor Ikaros staining, cells were stained with fluorochrome-labeled primary Abs for 20 min on ice. After washing in FACS buffer (BD Biosciences), cells were fixed with Fix/Perm™ Buffer (BD Biosciences). Following fixation, cells were permeabilized with Perm/Wash™ Buffer (BD Biosciences) and incubated with rabbit anti-mouse Ikaros (ab26083, Abcam, Cambridge, MA). Following staining with the Ikaros Ab, cells were washed and then stained with a PE-labeled anti-rabbit secondary Ab.

For phenotypic and functional analysis PBNs, BMNs and TANs were gated on live $CD11b^{+}CD15^{hi}CD66b^{+}$cells. The following cell surface antibodies were utilized: anti-CD11b (Biolegend, clone: ICRF44), anti-CD15 (Biolegend, clone: HI98), anti-CD66b (Biolegend, clone: G10F5), anti-CD54 (Biolegend, clone: HA58), anti-CD62L Biolegend, (clone: DREG-56), anti-CCR5 (Biolegend, clone: HEK/1/85a), anti-CCR7 (Biolegend, clone: G043H7), anti-CXCR1 (Biolegend, clone: 8F1/CXCR1), anti-CXCR2 (Biolegend, clone: 5e8/cxcr2), anti-PD-L1 (Biolegend, clone:M1H1), anti-Gal-9 (Biolegend, clone: 9M1-3), anti-CD301 (Biolegend, clone: H037G3), anti-CD200R, (Biolegend, clone: OX-108), anti-FASL (Biolegend, clone: NOK-1), anti-TRAIL (Biolegend, clone: RIK2), anti-TWEAK (clone: CARL-1), anti-CD86 (Biolegend, clone: IT2.2), anti-CD80 (Biolegend, clone:2D10), anti-CD40 (Biolegend, clone:5C3), anti-OX40L (Biolegend, clone:11C3-1), anti-4-1BBL (Biolegend, clone:5F4), anti-HLA-A2 (Biolegend, clone: bb7.2), anti-CD14 (Biolegend, clone: M5E2), anti-HLA-DR (BD Bioscience, clone: G46-6), anti-CD206 (Biolegend, clone: 15-2), anti-CD115 (Biolegend, clone: 9-4D2-1E4), anti-CD83 (Biolegend, clone: HB15e), anti-CD1c (Biolegend, clone: L161), anti-CD204 (Biolegend, clone: 7G5C33), anti-CD209 (Biolegend, clone: 9E9A8), anti-CD163 (Biolegend, clone: GHI/61). All data were acquired using the BD FACSCalibur or BD LSRFortessa™ (BD Bioscience) flow cytometers and analyzed using FlowJo software (TreeStar Inc.).

Antigen non-specific T cell response

To induce antigen non-specific T cell responses, PBMC or purified T cells were stimulated with plate-bound anti-human CD3 and/or anti-CD28 antibodies. To evaluate the effects of different neutrophil subsets on antigen non-specific autologous T cell response we measured: (i) the T cell proliferation using standard CFSE dilution method or BrdU incorporation assay, (ii) the T cell IFN-γ production using intracellular cytokine staining, and (iii) the expression of T cell activation markers CD25 and CD69 using flow cytometry.

PBMCs or purified T cells (responders) were labeled with 5 μM of the fluorescent dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Molecular Probe), according to the manufacturer's instructions. CFSE-labeled PBMCs or T cells were stimulated with plate-bound anti-human CD3 Ab or anti-human CD3 (clone: OKT3) and anti-human CD28 (clone: CD28.2) Abs (Biolegend), respectively. To coat the 96 U-bottom well plates with Abs, anti-CD3 (1 μg/ml) and/or anti-CD28 Abs (5 μg/ml) were added in 100 μL of PBS per well and incubated for 4 hours at 37°C. Wells were washed twice with PBS before the addition of cells. CFSE-labeled responders ($1.5x10^{5}$ cells /well) were mixed with either different subsets of TANs or differentiated BMNs or PBNs in a 1:1 ratio and co-cultured in CD3/CD28-coated plates for 4 days in the complete cell culture media. The CFSE signal was analyzed by flow cytometry on gated CD4 and CD8 lymphocytes. In other experiments, the proliferation of T cells was assessed by flow cytometry using BrdU Flow Kit (BD Pharmingen). Forty-eight hours after stimulation, T cells were exposed to bromo-deoxyuridine for 12 hours.

To measure the IFN-γ production, $1.5x10^{5}$ of autologous PBMC stimulated with plate-bound anti-human CD3 Ab were co-incubated with different neutrophil subsets in a 1:1 ratio for 48 hr in 96 well U-bottom plate in the complete cell culture media. To accumulate intracellular IFN-γ BD GolgiStop™ and BD GolgiPlug™ were added into the cell cultures during the last 12 hr. The cells were collected, washed in Stain Buffer (BD Biosciences) and stained for surface markers as described above. Surface stained cells were fixed with BD Cytofix™ Fixation Buffer (BD Biosciences) for 20 minutes. The fixed cells were permeabilized with BD Perm/Wash™ Buffer (BD Biosciences) and then stained with the anti-human IFN-γ (Biolegend, clone: 4S.B3). The percent of IFN-γ positive CD4 and CD8 cells was analyzed by flow cytometry.

To measure the expression of T cell activation markers, purified autologous T cells stimulated with plate-bound anti-human CD3 and CD28 Ab were co-incubated with different neutrophil subsets at

concentration $1.5x10^5$ cells/well (96 well U-bottom plate) in a 1:1 ratio for 24 hr in the complete cell culture media. The cells were collected, washed in Stain Buffer (BD Biosciences) and stained for surface activation markers CD25 and CD69 as described above.

Virus-specific memory T cell response

Autologous T cells purified from peripheral blood with human T cell enrichment columns (R&D Systems, Inc.) were used as responders and co-cultured with different subsets of neutrophils that had been pulsed with mixture of viral peptides from Cytomegalovirus, Epstein-Barr virus, Influenza virus or Clostridium tetani with a broad array of HLA types. Since most humans have been exposed to these antigens, these peptide pools are good control antigens for eliciting a response from antigen-specific memory T cells in PBMC samples. Specifically, TAN subsets were sorted based on the phenotype of canonical ($CD11b^+CD66b^+CD15^{hi}HLA\text{-}DR^-$) and hybrid ($CD11b^+CD66b^+CD15^{hi}HLA\text{-}DR^+$) TANs as described above. BM-derived canonical and hybrid neutrophils were differentiated with different types of TCM as described above. Tumor and BM-derived canonical and hybrid neutrophils were incubated with 2 μg/ml of PepMixCEF-MHC class I or PepMixCEFT-MHC class II peptide pools (JPT Peptide Technologies) for 30 minutes. Neutrophil subsets incubated with irrelevant mesothelin-derived peptides (FLLFSLGWV and YLIENVVTV) were used as a negative control to define a background. Following extensive washing, $1x10^4$ of neutrophils pulsed with viral peptides were incubated with $5x10^4$ autologous T cells in 96-Well PVDF Membrane ELISPOT Plate (Millipore) for 2 days. The T cell response was quantified by human IFN-gamma ELISPOT (Ready-SET-Go!®, ebioscience) according to the manufacturer's instructions. IFN-γ positive spots were counted and analyzed using ImmunoSpot® S5 Micro Analyzer (Cellular Technology Limited)

Generation of NY-ESO specific Ly95 T cells and A549-NY-ESO-1-A2 target lung cancer cell line

The NY-ESO-1–reactive Ly95 TCR construct is an affinity-enhanced variant of the wild-type IG4 TCR identified from T cells recognizing the HLA-A2 restricted NY-ESO-1:157-165 peptide antigen. The generation of this Ly95 TCR construct and its packaging into a lentiviral vector has been described in details earlier (Moon, et al, 2015; Robbins, et al, 2008). Human T cells were isolated from PBMC of healthy volunteer donors by negative selection using RosetteSep kits (Stem Cell Technologies, Vancouver, Canada). Isolated T cells were stimulated with magnetic beads coated with anti-CD3/anti-CD28 at a 1:3 cell to bead ratio. T cells were transduced with lentiviral vectors at an MOI of approximately 5. Cells were counted and fed with complete cell culture medium every 2 days. A small portion of expanded cells was stained for flow cytometry confirmation of successful Ly95 transduction using the Vβ13.1 TCR chain antibody (Beckman Coulter: clone IMMU 222). Transduction of human T cells undergoing anti-CD3/CD28 bead activation with high titer lentivirus that encodes the Ly95 TCR recognizing NY-ESO-1 resulted in approximately 20-50% of $TCRVb13.1^+$ CD8 cells.

For target cells, we genetically modified the A549 human lung adenocarcinoma cell line to express both NY-ESO-1 protein and HLA-A*02 as described earlier (Moon, et al, 2015). Briefly, A549 cell line was transduced by a retroviral vector encoding NY-ESO-1-T2A-HLA-A*02. The transduced A549 cells were subjected to limiting dilution at 0.5 cell per well in 96-well plates. Resulting clones were tested by flow cytometry for HLA- A*02 expression using anti-HLA-A2 Abs (Biolegend, clone: bb7.2). HLA-A2 positive clones were selected and tested in co-culture with T cells expressing the NY-ESO-1 Ly95 TCR. The clones expressing HLA-A2 that could stimulate NY-ESO-1 Ly95 TCR-expressing T cells to secrete IFN-γ were pooled to generate the A549-NY-ESO-1-A2 (A549-A2-ESO) cell line.

NY-ESO-specific T cell response

To study the regulation of antigen-specific effector T cell responses by neutrophil subsets, we used TCR transduced T cells (Ly95 T cells) recognizing the HLA-A2 restricted NY-ESO-1:157-165 peptide antigen (Moon, et al, 2015). In one set of experiments in order to stimulate the Ly95 T cell response we used A549 human lung adenocarcinoma cell line that was genetically modified to express both NY-ESO-1 protein and HLA-A*02 A549 (A2-NY-ESO-1 tumor cells). The Ly95 T cells at concentration $1.5x10^5$ cells/well (96 well-U-bottom plate) were mixed with A549 A2-NY-ESO-1 tumor cells in the presence of different neutrophil subsets at ratio 1:0.25:1 (Ly95 T cells : A549 A2-NY-ESO-1 : Neutrophils) for 18 hours in the complete cell culture media. BD GolgiStop™ and BD GolgiPlug™ were added into the cell cultures during the last 12 hr. The Ly95 T cells co-cultured with NY-ESO-1 negative A549 tumor cells and neutrophil subsets were used as a negative control to define the level of allostimulation. The cells were

collected, washed in Stain Buffer (BD Biosciences) and stained for CD8 and Ly95 TCR surface markers using anti-CD8 (Biolegend, clone: HIT8a) and anti-TCRVβ13.1 (Beckman Coulter: clone IMMU 222) antibodies with following intracellular staining for IFN-γ as described above. The production of IFN-γ and Granzyme B was analyzed in gated $CD8^+TCRV\beta13.1^+$ cells by flow cytometry.

In several experiments, blocking Abs against CD86 (clone: IT2.2), OX40L (clone: 11C3.1), 4-1BBL (clone: 5F4), CD54 (clone: HCD54), (all from Biolegend) were added to the co-cultures of hybrid neutrophils and Ly95 T cells activated with A549 A2-NY-ESO-1 tumor cells. The blocking Abs at the concentration 5 μg/ml were present in neutrophils/Ly95 cell co-culture for 18 hours, starting from the beginning of the assay. Matched isotype antibodies were used as controls. Transwell assays were performed using 24-well flat-bottom Transwell culture plates (Corning) with inserts of 0.4 μm membrane pore size (Corning). To separate Ly95 T cells and neutrophil subsets, 0.5×10^6 Ly95 T cells were mixed with A549 A2-NY-ESO-1 tumor cells in ratio 1:0.25 and added to the bottom chamber. BM-derived canonical and hybrid neutrophils were placed in the top at a ratio of 1:1 (Ly95 cells : Neutrophils). Cells were cultured in complete cell culture media for 24 hours and the production of intracellular IFN-γ was measured in gated $CD8^+TCRV\beta13.1^+$ cells by flow cytometry, as described previously.

Antigen-presenting cell functions of hybrid neutrophils

To assess whether the hybrid neutrophils perform function of APC, the effector Ly95 T cells were stimulated with different subsets of HLA-A*02$^+$ neutrophils pulsed with HLA-A*02-restricted NY-ESO-1 (157-165, SLLMWITQV) peptide. For this purpose, HLA-A*02 positive BM-derived canonical and hybrid neutrophils were incubated with NY-ESO-1 peptide (1 μg/ml) for 1 hour, washed three times with cell culture medium and mixed with Ly95 T cells at concentration $1.5x10^5$ cells/well (96 well U-bottom plate) in ratio 1:1 in the complete cell culture media. Eighteen hours later, NY-ESO-specific activation of the Ly95 cells was assessed by measuring intracellular IFN-γ in gated $CD8^+TCRV\beta13.1^+$ cells as described above.

To assess whether the hybrid neutrophil cross-present NY-ESO epitopes, HLA-A*02 positive BM-derived canonical and hybrid neutrophils were differentiated as described above but in AIM V AlbuMAX® serum free cell culture medium. These neutrophil subsets were incubated with free NY-ESO full-length protein (5 μg/ml) or NY-ESO immune complex for 12 hours in AIM V AlbuMAX® serum free cell culture medium prior Ly95 T cells assays. NY-ESO Immune complexes were formed by incubating the NY-ESO full-length protein (5 μg/ml) with monoclonal anti-NY-ESO Abs (clone E978, Thermo Scientific™) for 30 minutes at 37°C. Following extensive washing in serum free medium, $1x10^5$ neutrophils were mixed with $5 x10^3$ Ly95 T cells (transduction efficiency: 20% of $CD8^+TCRV\beta13.1^+$ cells) in 96-Well PVDF Membrane ELISPOT Plate (Millipore) in AIM V AlbuMAX® serum free cell culture. NY-ESO-free neutrophils incubated with Ly95 T cells were used as a negative control to define a background and level of allostimulation. Twenty four hours later, the NY-ESO-specific production of IFN-γ by Ly95 cells was assessed by human IFN-γ ELISPOT (Ready-SET-Go!®, ebioscience) according to the manufacturer's instructions. IFN-γ positive spots were counted and analyzed using ImmunoSpot® S5 Micro Analyzer
(Cellular Technology Limited).

To determine the ability of canonical and hybrid neutrophils to uptake and process an antigen, we used DQ-OVA (Molecular Probes) which is a self-quenched conjugate of ovalbumin that exhibits bright green fluorescence upon proteolytic degradation. Briefly, BM-derived canonical and hybrid neutrophil subsets were incubated with DQ-OVA (10 μg/ml) at 37°C for 2 hours. Cells incubated at 4°C served as controls. Neutrophils were collected, washed with cold Stain Buffer (BD Biosciences) and stained with APC-anti-HLA-DR Abs (BD Bioscience, clone: G46-6) at 4°C. The green fluorescence was analyzed by flow cytometry in canonical HLA-DR$^-$ and hybrid HLA-DR$^+$ neutrophils.

Allogeneic Mixed Lymphocyte Reaction (MLR)

Purified allogeneic T cells from healthy donor PBMCs were used as responders and reacted with $1 x10^5$ BM-derived canonical or hybrid neutrophils (inducers) from lung cancer patients at a ratio of 1:1 in 96-well round bottom plate (Corning®). Five days later, the proliferation of CD4 and CD8 T cells was measured using BrdU incorporation assay (BD Pharmingen) according to the manufacturer's instructions.

Phagocytosis

The phagocytic activity of neutrophil subsets was assayed with the pHrodo™ Red *E. coli* BioParticles® Phagocytosis Kit for flow cytometry (Life Technologies™), according to the manufacturer's instructions. Briefly, TANs or BM-derived hybrid neutrophils were incubated with pHrodo™ Red *E. coli* for 1 hour at 37°C in 5% CO2. After incubation, the neutrophils were washed twice with cold PBS and stained for the surface HLA-DR to distinguish the canonical HLA-DR^- and hybrid HLA-DR^+ neutrophils. The level of phagocytosis was analyzed by flow cytometry in gated HLA-DR^+ and HLA-DR^- cells.

Neutrophil survival in vitro

PBNs and BMNs were cultured at concentration 1 $x10^6$ /ml in the presence or absence of TCM (50% v/v) in 24 well clear tissue culture-treated plate (Corning®) in complete cell culture media. Three and 7 days later neutrophil viability was analyzed by flow cytometry using Fixable Viability dye FVD 660 (eBioscince).

Measurement of cytokines, chemokines and growth factors

The levels of 30 cytokines/chemokines and growth factors were measured in TCM using the Cytokine Human Magnetic 30-Plex Panel for the Luminex® platform (Invitrogen), according to the manufacturer's instructions. The concentration of IFN-γ and GM-CSF in TCM was measured with commercial ELISA kits purchased from BD Bioscience. Standards and samples were analyzed in triplicates and the mean value used for analysis.

TNF and IL-12 production by canonical and hybrid TANs were measured by intracellular staining after 6 and 24 hours of LPS stimulation (100 ng/ml), respectively. For intracellular cytokine staining, fixed TANs stained for HLA-DR were permeabilized with BD Perm/Wash™ Buffer (BD Biosciences) and then stained with the following Abs for 45 minutes at room temperature: APC anti-human TNF-α (Biolegend, clone: MAB11) and PE anti-human IL-12 (Biolegend, clone C11.5).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tumor specimens collected at the time of surgical resection were co-stained for neutrophils (MPO) and antigen-presenting cells (HLA-DR) using antibodies against HLA-DR (Biolegend; Clone L243, 1:12,000 dilution), CD66b (BD Biosciences: clone G10F5, 1:1000 dilution), and against human myeloperoxidase (MPO) (Dako; Polyclonal; 1:6000 dilution). Secondary staining was done using Leica Bond refine detection polymer (DAB) or Refine Red detection (Alk Phosphatase). All staining was performed on an automated stainer Bond III (Leica Biosystems Inc, Richmond VA).

Statistics

All data were tested for normal distribution of variables. Comparisons between two groups were assessed with a 2-tailed Student's *t*-test for paired and unpaired data if data were normally distributed. Non-parametric Wilcoxon matched-pairs test and Mann-Whitney unpaired test were used when the populations were not normally distributed. Likewise, multiple groups were analyzed by one-way analysis of variance (ANOVA) with corresponding Tukey's multiple comparison test if normally distributed, or by the Kruskal–Wallis with Dunn's multiple comparison test if not normally distributed. Non-parametric Spearman test was used for correlation analysis. All statistical analyses were performed with GraphPad Prism 6. A p value <0.05 was considered statistically significant.

Supplemental References

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