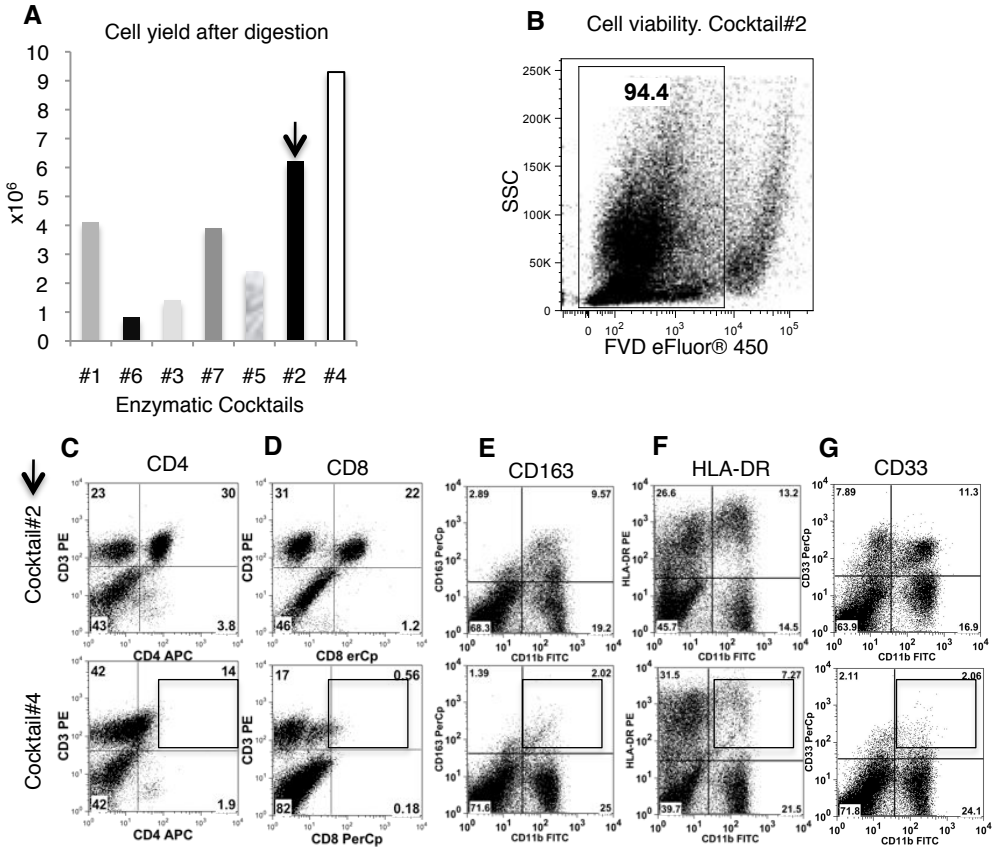
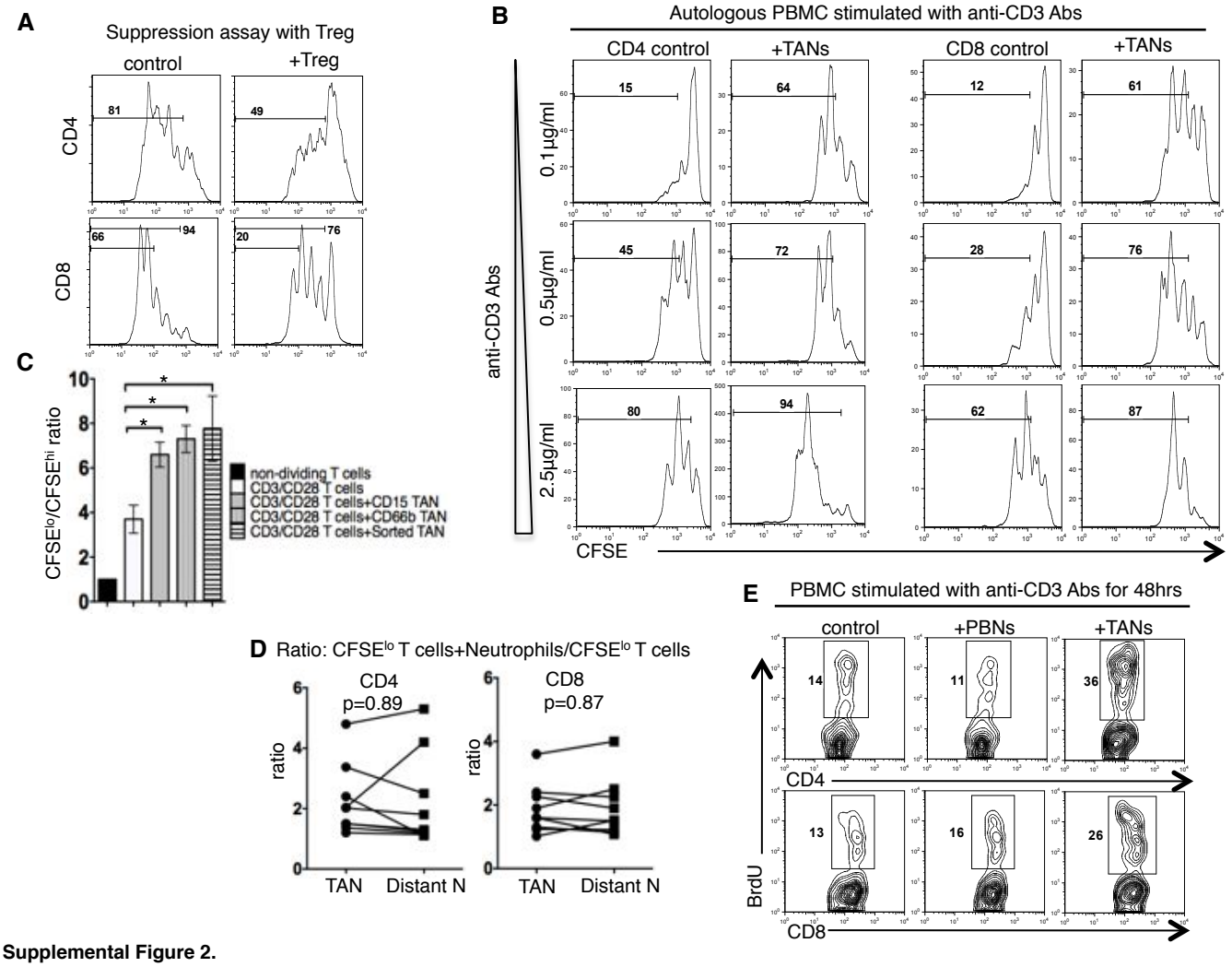
Supplemental figures and tables

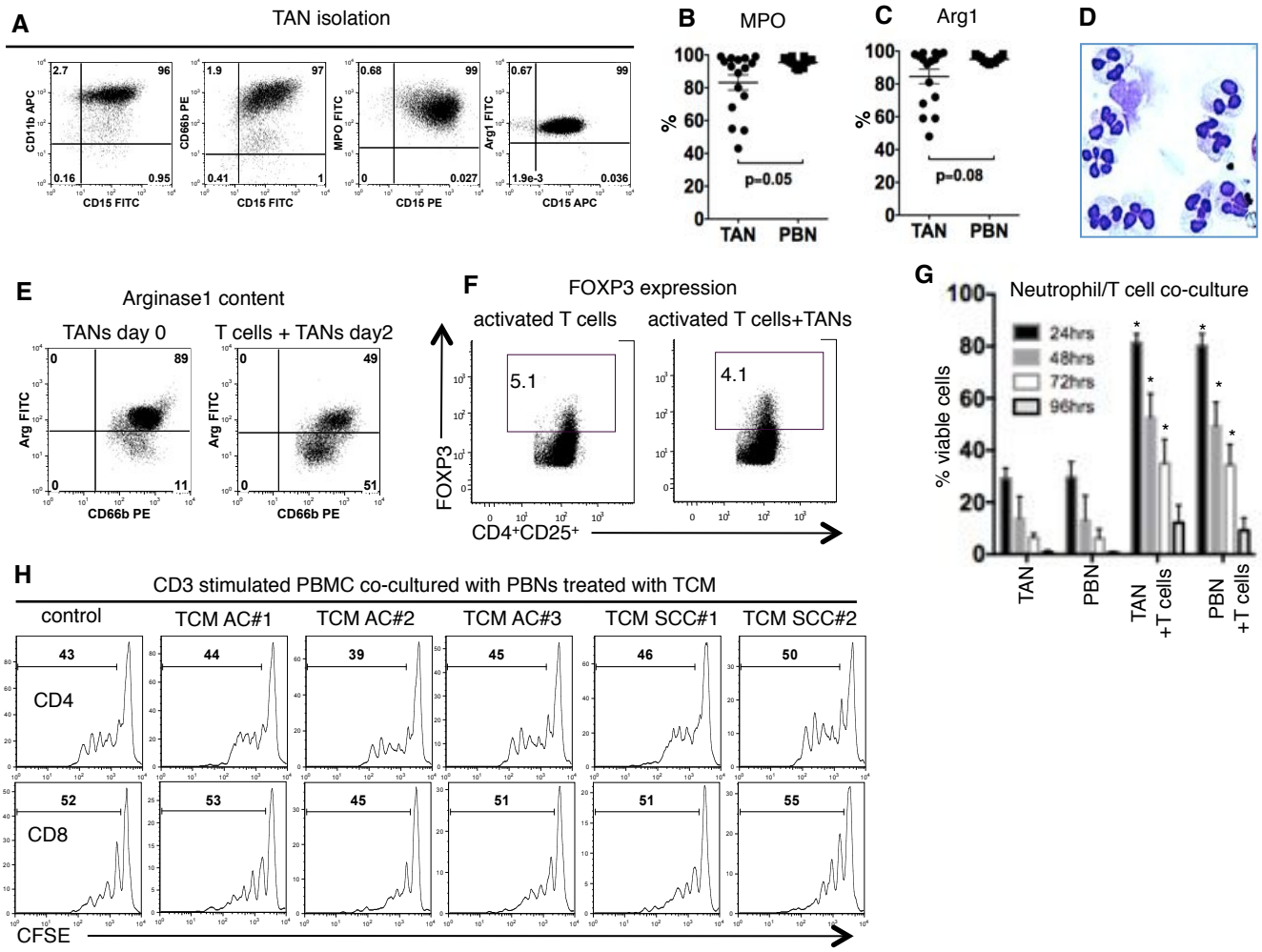


Supplemental Figure 1.
The digestion efficiency of different enzymatic cocktails recipes. (**A**) Viable cell yield after the digestion of tumor tissue with different enzymatic cocktails. Arrow indicates our new designed enzymatic cocktail (Cocktail#2). The highest cell yield was observed after digestion of tumor tissue with enzymatic Cocktail#4 containing high concentration of collagenase and protease. Representative data from 1 of 3 experiments are shown. (**B**) Digested cells were stained with Fixable Viability Dye eFluor® 450 to detect dead/live cells. Representative dot plot from 1 of 15 experiments is shown. (**C-G**) Comparative effect of Cocktail#2 and Cocktail#4 on the cleavage of CD4, CD8, CD163, HLA-DR and CD33 receptors in tumors dissociates after digestion. Tumor tissue was digested with different enzymatic cocktails and the expression of lymphoid and myeloid cell surface markers was analyzed on $CD45^+$ gated cells by flow cytometry. Significant cleavage of the indicated lymphoid and myeloid cell markers by Cocktail#4 is shown on dot plots in the upper right quadrants (bottom row). For all dot plots numbers represent the percentage of cells in each quadrant. Representative dot plots from 1 of 5 experiments are shown.



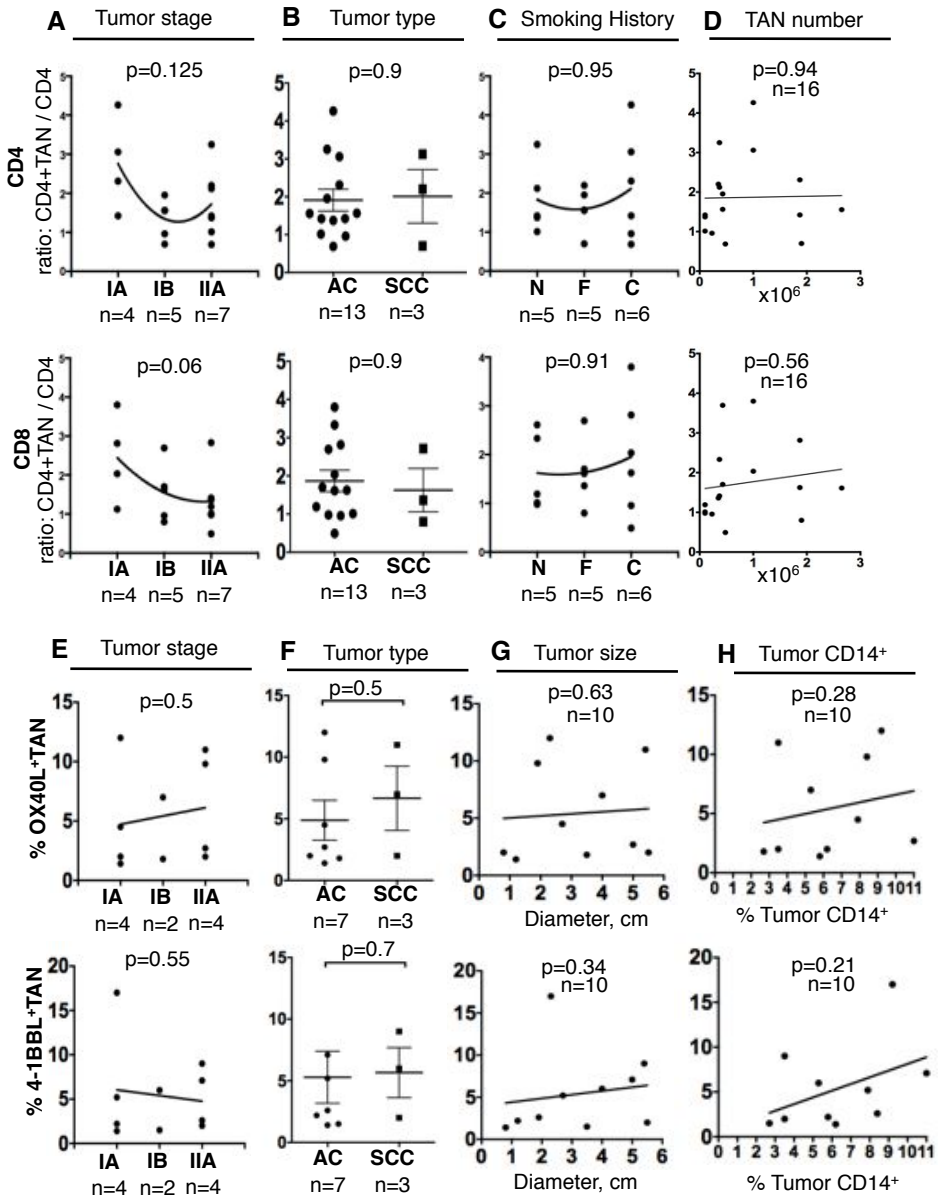
Supplemental Figure 2.

Flow cytometric analysis of T cell proliferation in the presence of TANs or Tregs. (**A**) Tregs were isolated from the PBMC of lung cancer patients and co-cultured with plate-bound anti-CD3 Abs activated PBMC from healthy donors at ratio 1:1 for 4 days. Representative results from 1 of 3 experiments are shown. (**B**) Autologous PBMC were stimulated with the indicated concentration of anti-CD3 Abs and co-cultured with TANs at ratio 1:1 for 4 days. Representative results from 1 of 3 experiment are shown. Numbers on all histograms represent the percentage of proliferating $CFSE^{lo}$CD4 and CD8 cells. (**C**) The stimulation of the T cell proliferation by TANs isolated using anti-CD15 beads, anti-CD66b beads or flow sorting. The ratio of the MFI of $CFSE^{lo}$ dividing cells to the MFI of $CFSE^{hi}$ non-dividing cells is shown. Error bars represent the mean ± SEM of 3 independent experiments, (* $p \leq 0.05$. Mann Whitney rank sum test). (**D**) Comparison of T cell stimulatory activity of TANs and distant neutrophils. Stimulatory activity of tissue neutrophils defined as ratio $CFSE^{lo}$ (T cells+Neutrophils) / $CFSE^{lo}$ (T cells). Summary results from 8 lung cancer patients are shown, Wilcoxon matched-pairs rank test. (**E**) Autologous T cells were stimulated with plate-bound anti-CD3 Abs and anti-CD28 Abs and cultured alone (control) or with PBNs or with TANs. Forty eight hours later, the proliferation of T cells was assessed by incorporation of BrdU into DNA. Numbers represent the percentage of cells in each quadrant. Representative results from 1 of 3 experiments are shown.

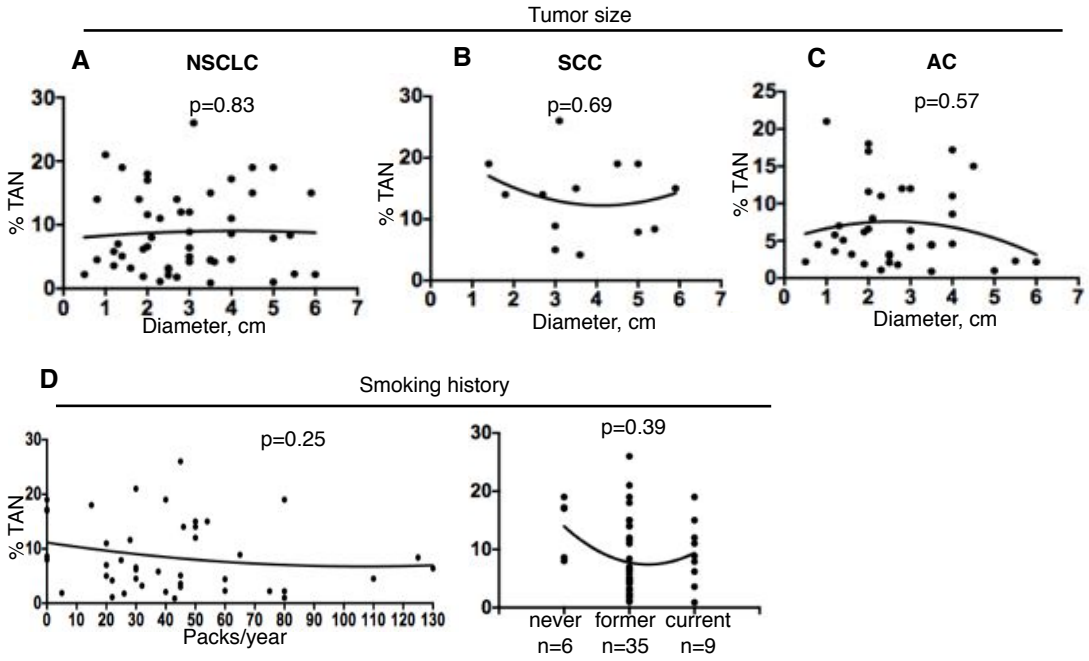


Supplemental Figure 3

The characterization of TANs. (**A**) The expression of CD11b, CD15, CD66b, as well as the intracellular MPO and Arg1 shows the purity of the isolated neutrophils. Representative dot plots from 1 of 10 experiments are shown. (**B** and **C)** Distributional dot plots of the MPO (n=16) and Arg1 content (n=16) in TANs and PBNs. Student's *t-test* for paired data. (**D**) Cytomorphology of purified TANs. (**E**) The Arginase 1 content in TANs co-cultured with activated autologous T cells for 2 days. Representative dot plots from 1 of 7 experiments are shown. (**F**) Allogeneic PBMC were stimulated with plate bound anti-CD3 Abs and mixed with TANs for 4 days. Intracellular FOXP3 expression was measured in the gated $CD25^{+}CD4^{+}$ cells. Representative results from 1 of 3 experiments are shown. Numbers on all dot plots represent the percentage of cells in each quadrant (**G**) Dynamic of the neutrophil survival in the co-culture with activated T cells. Fixable Viability dye was used to discriminate viable CD15 neutrophils. Error bars represent the mean ± SEM. Summary results from 6 cancer patients are show in the graph. *p≤0.001, significant differences between TANs and PBN cultured alone and with activated T cells at indicated time points, Kruskal-Wallis and Dunn's Multiple comparison tests. (**H**) Proliferation of autologous CD4 and CD8 cells in the presence of PBNs pre-treated with TCM. Numbers on all histograms represent the percentage of proliferating CD4 and CD8 cells. Representative results from 1 of 6 experiments are shown.



Supplemental Figure 4. Correlation analysis of the stimulatory activity of TANs in lung cancer patients (n=16) with different tumor stage (**A**), type (**B**), smoking history (**C**) and number of isolated TANs (**D**). Stimulatory activity of TANs defined as the ratio $CFSE^{lo}$ (T cells+TAN) / $CFSE^{lo}$ (T cells). Correlation analysis of the accumulation of $OX\text{-}40L^{+}$TANs and $4\text{-}1BBL^{+}$TANs in lung cancer patients (n=10) with different tumor stage (**E**) type (**F**), size (**G**) and number of intratumoral CD14 cells (**H**). Non-parametric Spearman test was used to determine the degree of correlation. Two groups were analyzed using Mann-Whitney test. Error bars represent the mean ± SEM.



Supplemental Figure 5. Correlation analysis of the TAN frequency in patients with NSCLC with different tumor size (n=50) (**A**), in patients with AC (n=37) (**B**) or SCC (n=13) (**C**). (**D**) Correlative analysis of the TAN frequency with smoking history and consumption (packs per year) in patients with NSCLC (n=50). Statistical analyses were performed using non-parametric Spearman correlation.

Supplemental Table 1: Patient Characteristics (n=86)

Cancer Type	
Adenocarcinoma	58
Squamous Cell Carcinoma	16
Other histology	12
Age	
Median	66.0
Average	66.7
Range	25-85
Sex	
Male	54
Female	32
Race	
White	55
Black	23
Hispanic	3
Unknown	5
Tumor Stage	
Stage IA	34
Stage IB	27
Stage IIA	16
Stage IIB	6
Indeterminable	3
Tumor Grade	
T1a	27
T1b	12
T2a	30
T2b	10
T3	5
Indeterminable	2
Nodal Stage	
N0	75
N1	9
Indeterminable	2
Smoking History	
Current	20
Former	54
Never	8
Unknown	4

Supplemental Table 2: Comparative analysis of the TAN characteristics in lung cancer patients with different clinicopathological parameters

TAN	tumor type		tumor stage		tumor size		smoking history		
	AC	SCC	I	II	<3cm	>3cm	N	F	C
frequency	7.1 ± 0.9 (n=39)	13.4 ± 1.7 (n=11)	9.1 ± 1.1 (n=33)	9.3 ± 2.6 (n=17)	8.3 ± 1.1 (n=26)	9.2 ± 1.3 (n=24)	7.7± 1.1 (n=6)	13.9± 2.3 (n=35)	9.4± 1.9 (n=9)
	p=0.001		p=0.95		p=0.61		p=0.9		
CD62L	29± 3.9 (n=11)	23± 9.1 (n=4)	28± 3.8 (n=12)	26± 10.8 (n=3)	32± 5.8 (n=7)	24± 4.8 (n=8)	31± 14.1 (n=2)	25± 4.1 (n=11)	38± 5.1 (n=2)
	p=0.67		p=0.85		p=0.21		p=0.48		
CD54	71± 5 (n=9)	82± 6.3 (n=4)	73± 5 (n=11)	84± 5 (n=2)	67± 6.4 (n=6)	81± 5.7 (n=7)	74± 10 (n=2)	74± 5.5 (n=9)	78± 10.5 (n=2)
	p=0.2		p=0.5		p=0.07		p=0.89		
CCR5	75± 6.4 (n=11)	56± 7.4 (n=5)	70± 6.3 (n=10)	68± 10 (n=6)	71± 4.9 (n=9)	67± 11 (n=7)	77 (n=1)	72± 5.2 (n=14)	28 (n=1)
	p=0.04		p=0.77		p=0.89		N/A		
CXCR3	18± 3.6 (n=16)	19.5± 7.5 (n=3)	20± 4.5 (n=14)	11± 3 (n=5)	22± 6 (n=10)	13± 2.8 (n=9)	7.1± 4.9 (n=2)	23± 4.8 (n=12)	7.8± 1.9 (n=5)
	p=0.67		p=0.51		p=0.52		p=0.03		
OX40L	4.7± 1.6 (n=7)	6.3± 2.3 (n=3)	4.8± 1.7 (n=6)	6.4± 2.3 (n=4)	5.9± 2.1 (n=5)	4.9± 1.7 (n=5)	-	5.5± 1.4 (n=7)	5.1± 3.5 (n=3)
	p=0.55		p=0.5		p=0.89		p=0.7		
4-1BBL	5.2± 2.1 (n=7)	5.6± 2 (n=3)	5.5± 2.4 (n=6)	5.1± 1.7 (n=4)	5.6± 2.9 (n=5)	5.1± 1.4 (n=5)	-	4.7± 1.1 (n=7)	7± 4.9 (n=3)
	p=0.67		p=0.55		p=0.8		p=0.9		
CD86	6.4± 1.3 (n=17)	4.9± 2.3 (n=6)	6± 1.4 (n=15)	5.9± 2 (n=8)	6.1± 1.5 (n=14)	5.8± 1.9 (n=9)	3.5± 2.1 (n=2)	7.1± 1.4 (n=16)	3.3± 1.9 (n=5)
	p=0.54		p=0.76		p=0.86		p=0.1		
stimulation of CD4 cells	1.9± 0.3 (n=13)	2± 0.7 (n=3)	1.9± 0.4 (n=9)	1.7± 0.3 (n=7)	2.5± 0.4 (n=7)	1.3± 0.1 (n=9)	1.8± 0.4 (n=5)	1.6± 0.2 (n=5)	2.1± 0.5 (n=6)
	p=0.9		p=0.58		p=0.04		p=0.95		
stimulation of CD8 cells	1.8± 0.2 (n=13)	1.6± 0.5 (n=3)	2.1± 0.3 (n=9)	1.2± 0.2 (n=7)	2.1± 0.3 (n=7)	1.2± 0.1 (n=9)	1.6± 0.3 (n=5)	1.6± 0.3 (n=5)	1.9± 0.5 (n=6)
	p=0.71		p=0.11		p=0.04		p=0.91		

Statistical analyses were performed with Student *t-tests* for unpaired data to compare frequency of TANs in different groups. Comparisons between the other TAN characteristics were assessed using non-parametric Mann-Withey test. Multiple groups was compared using nonparametric Kruskal-Wallis test. Differences were statistically significant if the p-value was less than 0.05. The frequency of TANs determined by flow cytometry as the percentage of $CD11b^{+}CD15^{hi}CD66b^{+}$ cells among all nucleated cells in digested tumor tissue. Results are expressed as the mean percentage of $CD62L^{+}$, $CD54^{+}$, $CCR5^{+}$, $CXCR3^{+}$, $OX40L^{+}$, 4-1BBL^{+}, $CD86^{+}$ TAN ± standard error of the mean (SEM). Stimulatory activity of TANs defined as the ratio $CFSE^{lo}$ (T cells+TAN) / $CFSE^{lo}$ (T cells). Statistically significant differences between groups are highlighted in grey. N-never smoked, F-former smokers, C-current smokers.

Supplemental Methods

Detailed descriptions of all reagents and methods are provided below.

Reagents

Complete cell culture media DME/F-12 1:1 media (HyClone, Thermo Scientific) was supplemented with 2.5 mM L-glutamine, 15mM HEPES Buffer, 10% of Embryonic Stem (ES) Cell Screened FBS (U.S.) (Thermo Scientific™ HyClone™), Penicillin (100U/ml) and Streptomycin (100 µg/mL).

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. The T regulatory cells (Tregs) were isolated from the PBMCs of lung cancer patients with the human $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec, Inc).

Preparation of a single-cell suspension from lung tumor tissue

Surgically-removed fresh lung tumors from patients were processed within 20 minutes of removal from the patient. Under sterile conditions, all areas of tissue necrosis were trimmed away. The tumor 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 70μM 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 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% (**Supplemental Fig. 1B**). 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, TANs and PBNs were prepared at room temperature (RT) and rapidly utilized.

TANs: A single cell suspension was obtained by enzymatic digestion of tumor tissue. TANs were isolated from tumor cell suspensions using positive selection of $CD15^+$ or $CD66b^+$ cells with microbeads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). 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-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 $CD45^+CD11b^+CD66b^+CD15^+$. Sterile cell sorting was performed on the BD FACSAria II

(BD Biosciences, San Jose, USA). Neutrophils from distant non-involved lung tissue were isolated similarly to TAN.

PBNs: EDTA anti-coagulated peripheral blood was collected from lung cancer patients during surgery or from healthy donors. The granulocytes 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. The purity and activation status of isolated TANs 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. The TANs demonstrated high cell viability with minimal enzyme-induced premature cellular activation or cleavage of myeloid cell markers (**Supplemental Fig. 1**). The purity of TANs and PBNs was typically higher than 94%. Isolates with less than 90% purity were discarded. To evaluate the cytomorphology of isolated TANs and PBNs, cells were spun on glass slides and stained with the Hema3 Stat Pack Kit (Fisher Scientific).

Flow Cytometry

Flow cytometric analysis was performed according to standard protocols. Matched isotype antibodies were used as controls. To exclude dead cells from analysis, cells were stained with the Fixable Viability Dye eFluor® 450 (e-bioscience), Fixable Aqua Dead

Cell Stain Kit (Invitrogen) or Zombie Yellow™ Fixable Viability dye (Biolegend). For intracellular staining, fixed cells 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), or PE-anti-human IL-10 (Biolegend, clone: JES3-9D7). Intracellular staining of activated T cells for FoxP3 was performed with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and anti-human FoxP3 Abs (eBioscience, clone: PCH101) according to the manufacturer's instructions. The following antibodies were used: anti-CD11b (clone: ICRF44, Biolegend), anti-CD15 (clone: W6D3, BD Biosciences), anti-CD66b (clone: G10F5, Biolegend), anti-CD54 (clone: HA58, Biolegend), anti-CD62L (clone: DREG-56, Biolegend), anti-CCR1 (clone: TG4/CCR1, Biolegend), anti-CCR2 (clone: FAB151P, R&D systems), anti-CCR3 (clone: 5 E8, Biolegend), anti-CCR4 (clone:TG6/CCR4, Biolegend), anti-CCR5 (clone: HEK/1/85a, Biolegend), anti-CCR6 (clone: G034E3, Biolegend), anti-CCR7 (clone: G043H7, Biolegend), anti-CCR8 (clone: 191704, R&D systems), anti-CXCR1 (clone: 8F1/CXCR1, Biolegend), anti-CXCR2 (clone: 5e8/cxcr2, Biolegend), anti-CXCR3 (clone: G025H7, Biolegend), anti-CXCR4 (clone: 12G5, Biolegend), anti-CXCR7 (clone: 8F11-M16, Biolegend), anti-CD64 (clone:10.1, Biolegend), anti-CD32 (clone:FUN-2, Biolegend), anti-CD16 (clone:3G8, Biolegend), anti-CD89 (clone: A59, Biolegend), anti-PD-L1 (clone:M1H1, BD Biosciences), anti-Gal-9 (clone: 9M1-3, Biolegend), anti-CD301 (clone: H037G3, Biolegend), anti-CD200R, (clone: OX-108, Biolegend), anti-FASL (clone: NOK-1, Biolegend), anti-TRAIL (clone: RIK2, Biolegend), anti-TWEAK (clone: CARL-1, Biolegend), anti-CD86 (clone: IT2.2, Biolegend), anti-CD80 (clone:

2D10, Biolegend), anti-CD40 (clone: 5C3, Biolegend), anti-OX40L (clone: 11C3-1, Biolegend), anti-4-1BBL (clone: 5F4, Biolegend).

The data were acquired using the BD FACSCalibur or BD LSRFortessa^{TM} (BD Bioscience) flow cytometers and analyzed using FlowJo software (TreeStar Inc.).

T cell proliferation assay

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 (1μg/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 were mixed with either Tregs, TANs 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 several experiments, blocking Abs against CD86 (clone: IT2.2), CD80 (clone: 2D10), OX40L (clone: 11C3.1), 4-1BBL (clone: 5F4), CD54 (clone: HCD54), CD40 (clone: 5C3) (all from Biolegend) were added to the co-cultures of TANs and activated T cells at the concentration 1μg/ml. The blocking Abs were present in TAN/Tcell co-culture for 4 days, starting from the beginning of the proliferation assay. 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 18 hours. 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 T cells and TANs, 0.5×10^6 CFSE-labeled T cells were added to the CD3/CD28-coated bottom chamber and TANs were placed in the top at a ratio of 1:1. Cells were cultured in complete cell culture media for 4 days and the level of CD4 and CD8 proliferation was measured, as described previously.

Allogeneic Mixed Lymphocyte Reaction (MLR)

Purified allogeneic T cells from healthy donor PBMCs were used as responders and reacted with irradiated, mature, monocyte-derived DCs (MoDCs) (inducers) from unrelated healthy donors. Immature MoDCs were prepared by culturing adherent peripheral blood monocytes for 7 days in DMEM supplemented with 10% FBS, recombinant human GM-CSF (50ng/ml), and IL-4 (50ng/ml). To mature the MoDCs, LPS (100ng/ml) was added to the cell culture for 24 hours prior to harvest. The TANs or PBNs (regulators) were added to the DC-induced mixed lymphocyte reaction (MLR) as "third party cells" at a ratio of 1:0.25:1 (regulator: inducer: responder). Five days later, the proliferation of CD4 and CD8 T cells was measured using flow cytometric analysis of CFSE dilution.

Phagocytosis

The phagocytic activity of TANs and PBNs was assayed with the pHrodo™ Red *E. coli* BioParticles® Phagocytosis Kit for flow cytometry (Life Technologies™), according to the manufacturer's instructions.

Chemotaxis

We utilized a previously established protocol for fluorescence-based measurement of neutrophil migration *in vitro* (68). with minor modifications. Briefly, cells (5×10^6/mL) were labeled in HBSS with 2 μM calcein-AM (Life Technology). After washing with 10% FBS HBSS, $1x10^5$ cells were loaded in 25 μL HBSS onto the upper surface of the polycarbonate filter for each chemotaxis chamber of the 96-well ChemoTx® system (Neuro Probe, Gaithersburg, MD). The wells below the filter were filled with the appropriate concentrations of chemoattractant (50% v/v TCM, IL-8 and fMLP). Calcein AM-labeled-neutrophils at different concentrations were added in 30 μL to the bottom wells to produce a standard curve. The fluorescence of the cells that had migrated was measured in the GloMax® Multimode reader (Promega, Madison, WI). Cell migration was calculated as the percentage of maximum migration (cells added directly to the bottom of the wells), corrected for spontaneous migration.

Neutrophil Survival

Freshly isolated TANs or PBNs were cultured in complete cell culture media in the presence or absence of 50% v/v of TCM for 20 hours. Neutrophil viability, apoptosis, and necrosis were measured using the FITC-Annexin V Apoptosis Detection Kit (Biolegend) and analyzed by flow cytometry, according to the manufacturer's instructions.

Measurement of ROS

The production of H_2O_2 in TANs and PBNs isolated from lung cancer patients and healthy donors was measured using Amplex Red Hydrogen Peroxide/ Peroxidase Assay Kit (Invitrogen), according to the manufacturer's instructions. Briefly, 15,000 cells were incubated in HBSS in the presence of peroxidase and Amplex® Red reagent in Nunc™ F96 MicroWell™ Black Polystyrene Plate (Thermo Scientific). TANs or PBNs were stimulated with PMA (40ng/mL) or left unstimulated for 1 hour. Apocynin was added to a concentration of 250μM. The fluorescence of the oxidation product, resorufin, was measured using the green optical kit of the GloMax® Multimode reader (Promega, Madison, WI).

Measurement of cytokines, chemokines and growth factors

Single cell suspensions were obtained from lung tumors by enzymatic digestion, as described above. TANs were further isolated from the suspension. PBNs were isolated from lung cancer patients, as described above. Both unseparated cells and isolated neutrophils from digested tumors, and PBNs, were re-suspended in DME/F-12 1:1 medium with 10% FBS at a concentration of $1x10^6$ cells/mL. Twenty-four hours later, cell culture supernatants were collected, filtered, and stored at -80°C until measurement. The levels of 30 cytokines/chemokines and growth factors were measured using the Cytokine Human Magnetic 30-Plex Panel for the Luminex® platform (Invitrogen). The production of IFN-g, IL-10 and GM-CSF was measured with commercial ELISA kits

purchased from BD Bioscience. Standards and samples were analyzed in triplicates and the mean value used for analysis.

Immunohistochemistry

The Tumor Microarrays (TMAs) was constructed from formalin-fixed, paraffin-embedded tumor and adjacent normal specimens collected at the time of surgical resection. Sections from 45 adenocarcinoma and 25 squamous cell carcinoma patients were analyzed. After standard antigen retrieval, the TMAs were double stained with an anticytokeratin antibody (Novocastra; Clone AE1/AE3; 1:500 dilution) to label cancer cells and an antibody against human myeloperoxidase (MPO) (Dako; Polyclonal; 1:6000 dilution) to label neutrophils. Secondary staining was done using Leica Bond refine detection polymer (DAB) or Refine Red detection (Alk Phosphatase). Slide imaging was performed on a Vectra automated imaging robot (Perkin Elmer Inc, Waltham MA) and analyzed using Inform analysis software (Perkin Elmer Inc, Waltham MA). The digitized arrays were then quantitated to measure the number of hematopoetic cells (Red events) within areas of tumor (as identified by brown cytokeratin staining) or stroma (absence of cytokeratin staining) using Inform cytometric analysis. Data are expressed as the intraepithelial or stromal hematopoetic cell density per mm^2 of tumor tissue. In addition, we co-stained for neutrophils (MPO) and antigen-presenting cells using an antibody against HLA-DR (Biolegend; Clone L243, 1:12,000 dilution) and for neutrophils (MPO) and T cells lymphocytes using an anti-CD3 antibody (Novocastra; Clone LN10; 1:300 dilution). All staining was performed on an automated stainer Bond III (Leica Biosystems Inc, Richmond VA).