Supplemental material

Microenvironmental Th9– and Th17– lymphocytes induce metastatic spreading in lung cancer

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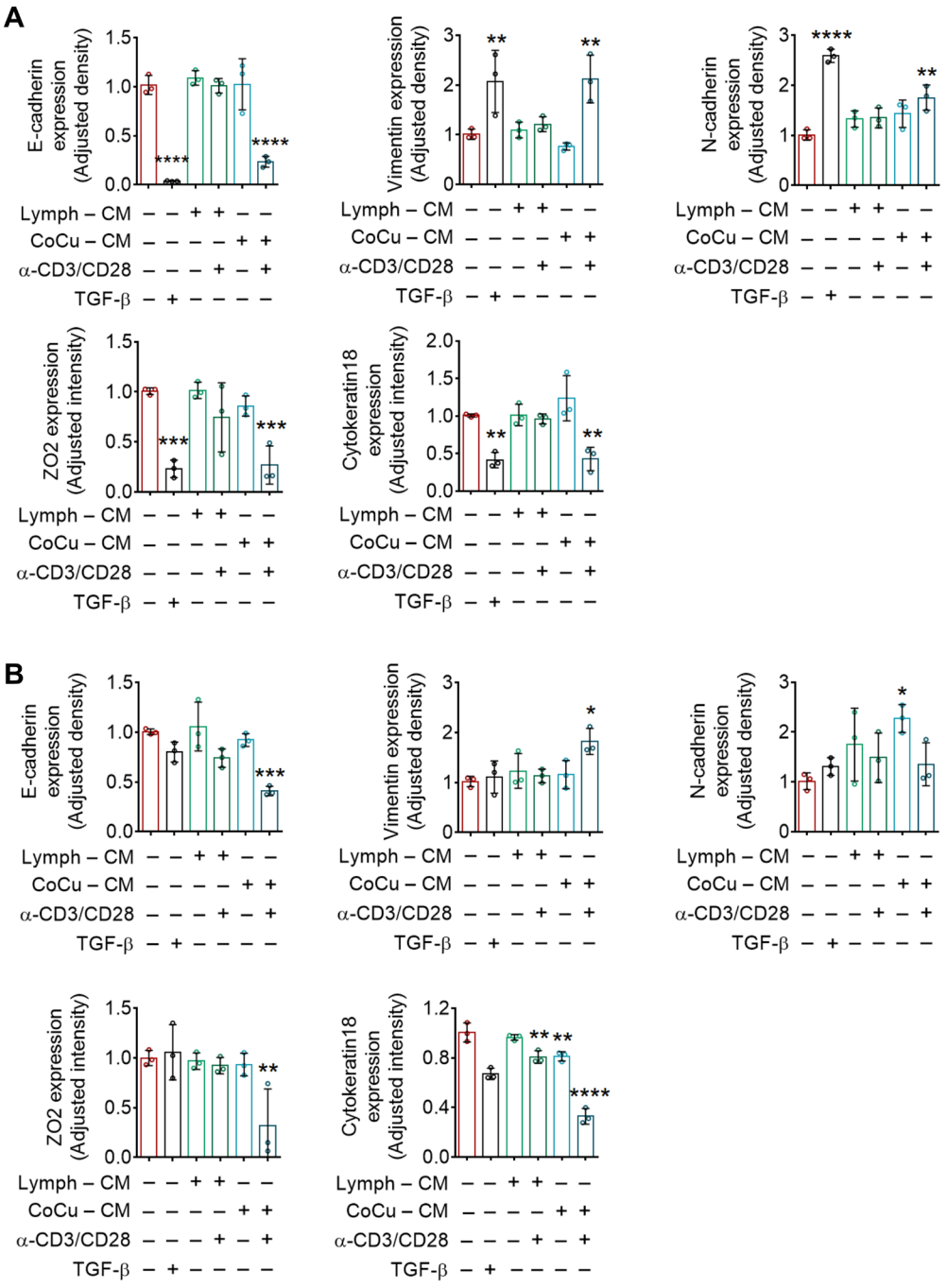
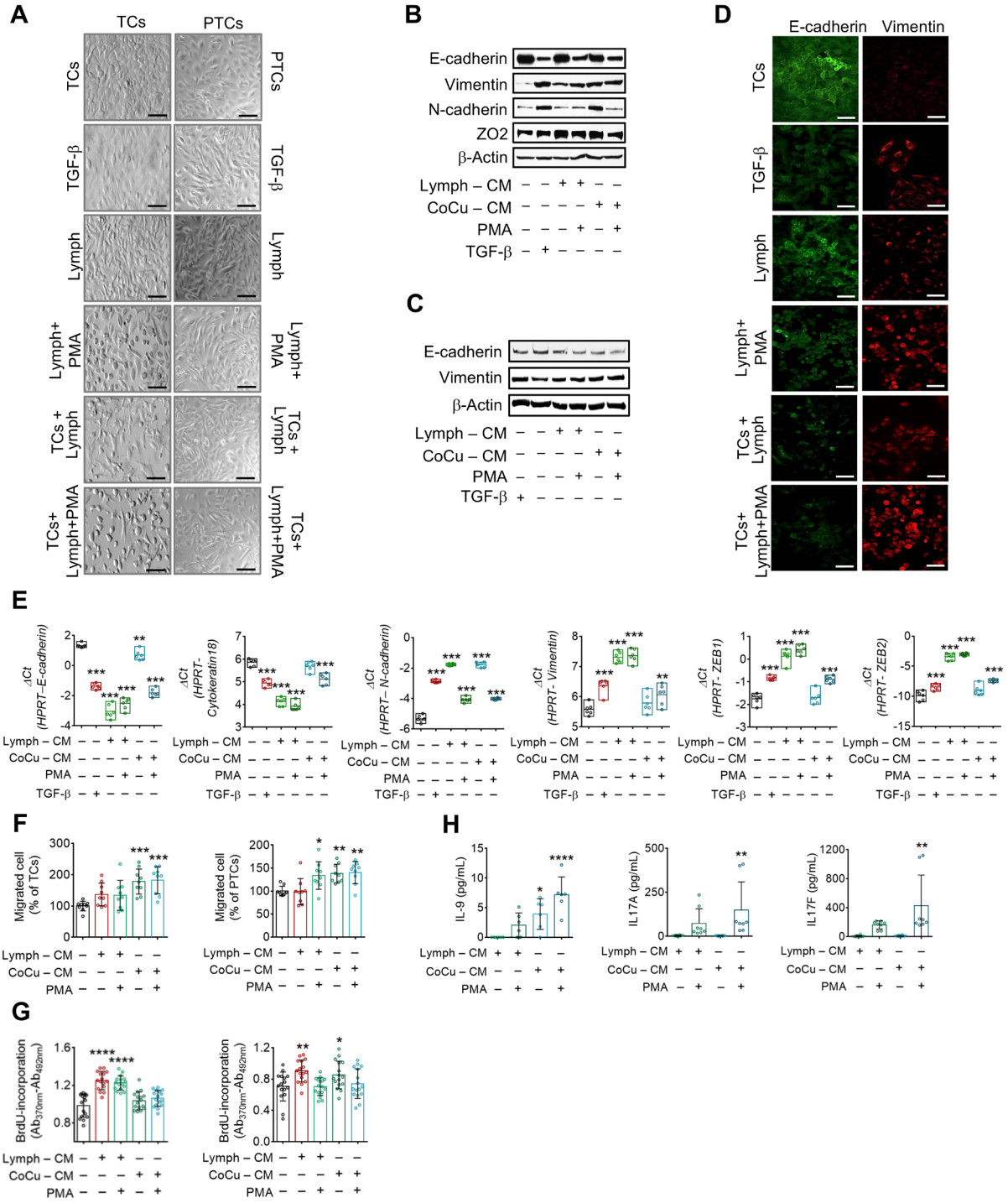


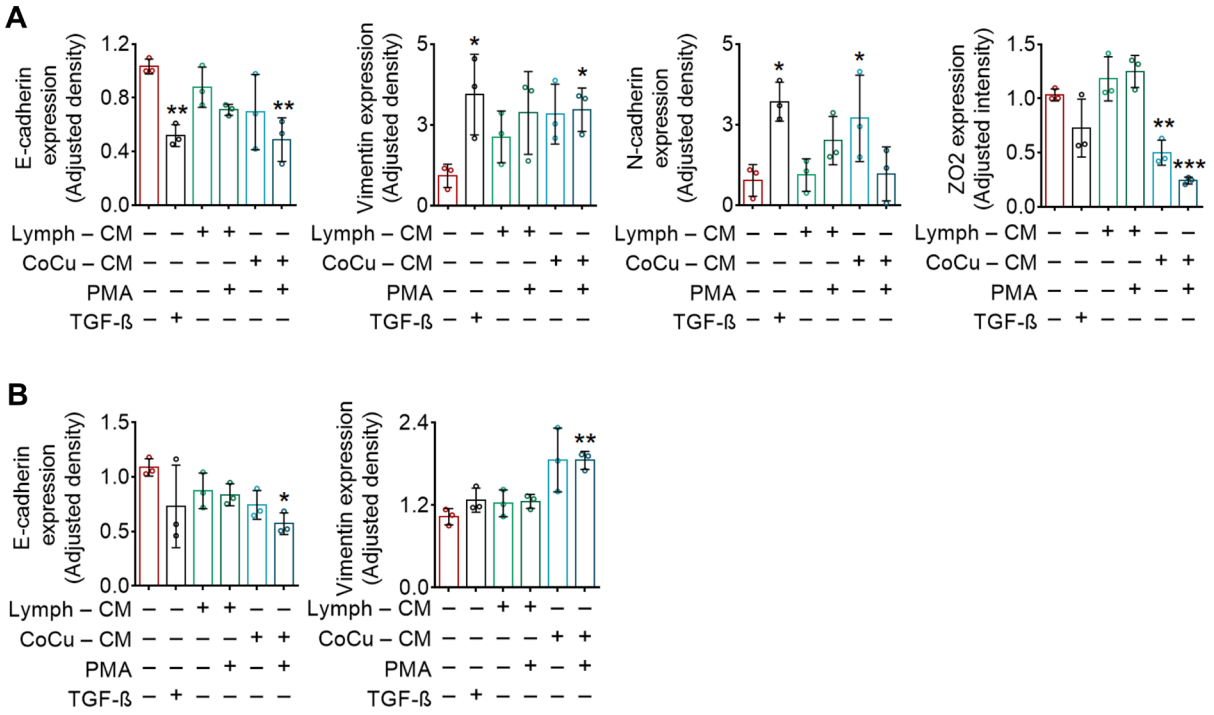
Supplementary Figure 1. Lymphocyte CM induces deregulation of EMT marker protein expression in human cancer cell lines and primary tumor cells: (**A**) Quantification of protein expression of EMT markers E-cadherin, Vimentin, N-cadherin, ZO2 and Cytokeratin18 after 48h stimulation of A549 cells. (**B**) Quantification of protein expression of EMT markers E-cadherin, Vimentin, N-cadherin, ZO2 and Cytokeratin18 after 48h stimulation of PTCs. (n = 3 donors) in

A and **B** **P < 0.01, ***P < 0.001 compared to A549 or PTCs using one-way ANOVA Dunnett's test.



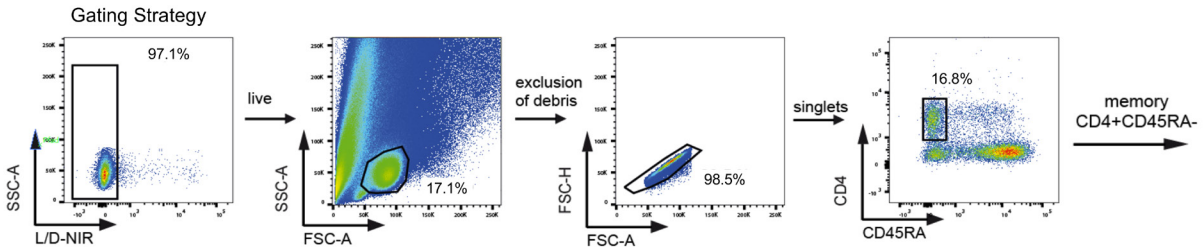
Supplementary Figure 2. PMA-activated Lymphocyte CM prompts EMT and enhances the migratory potential of human lung cancer cells: Conditioned medium (CM) of A549, primary human NSCLC cells (PTCs), Lymphocytes (Lymph -/+ PMA) or co-culture (A549+ Lymph) was

used for stimulation of A549 and PTCs to assess epithelial mesenchymal transition (EMT), migration and proliferation. (**A**) Representative photomicrographs depicting the morphology of tumor cells (A549 and PTCs) after 48h of stimulation with CM. (**B**) and (**C**) Western blot analysis of EMT markers (E-cadherin, Vimentin, N-cadherin and ZO2) from (**B**) A549 and (**C**) PTCs lysates after 48h stimulation with CM. (**D**) Immunocytochemistry of E-cadherin (green) and Vimentin (red) after 48h stimulation of A549 with CM. Scale bars: 50µm. (**E**) mRNA profile expression of EMT markers after 24h stimulation of A549. (n = 3 donors; 2 experimental replicates) (**F**) Migration and proliferation (as assessed by BrdU incorporation) of A549 after 6h and 24h of stimulation with CM, respectively. (**G**) Migration and proliferation (as assessed by BrdU incorporation) of PTCs after 12h and 48h of stimulation with CM respectively. (n = 3 donors 3 experimental replicates) (**H**) Quantitative analysis of IL-9 and IL-17A detected in CM with or without co-culture by ELISA (n=3 donors 2 experimental replicates). In E-H *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to A549, PTCs or Lymph-CM using one-way ANOVA Dunnett's test.

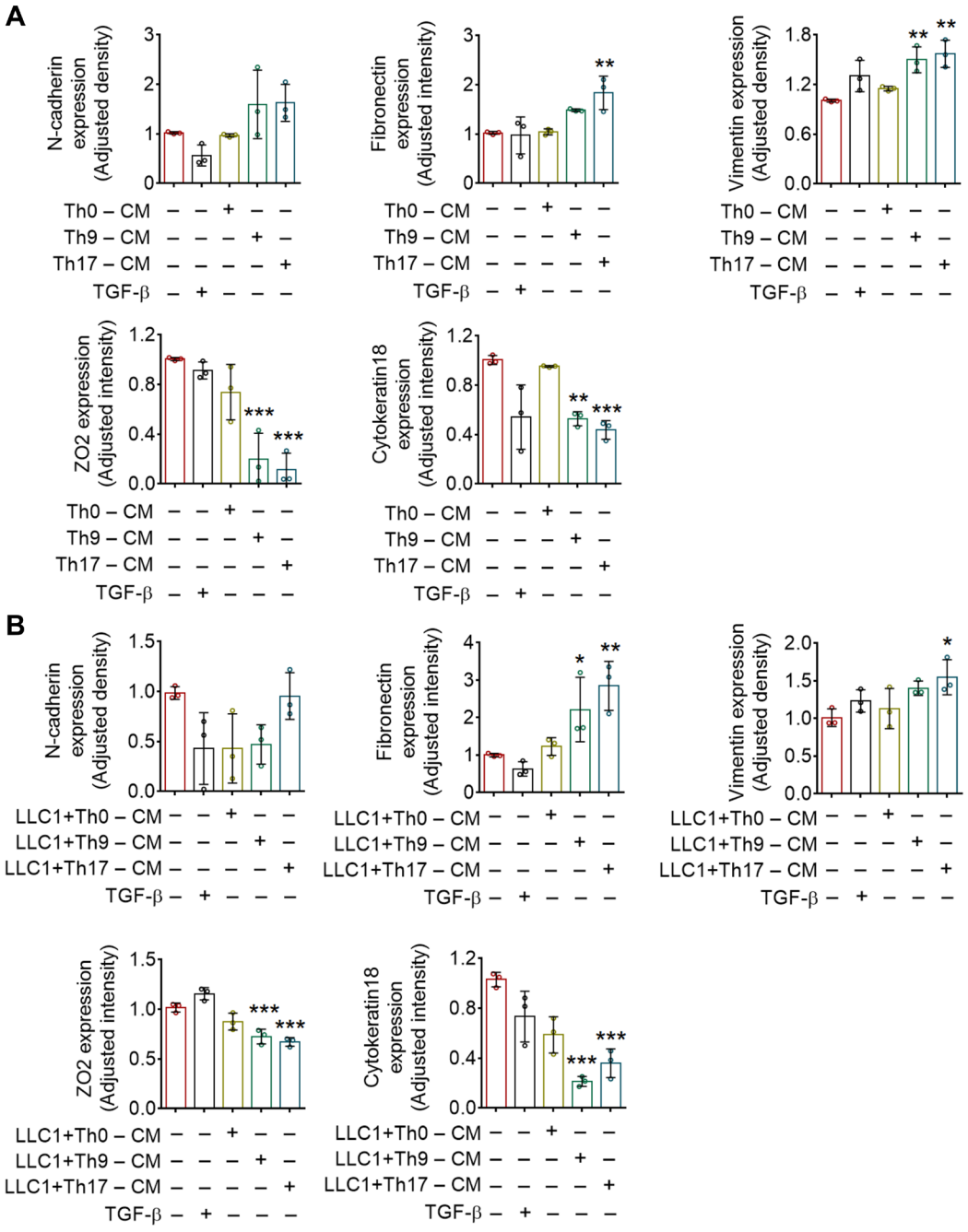


Supplementary Figure 3. Lymphocyte CM induces deregulation of EMT marker protein expression in human cancer cell lines and primary tumor cells: (**A**) Quantification of protein expression of EMT markers E-cadherin, Vimentin, N-cadherin and ZO2 after 48h stimulation of

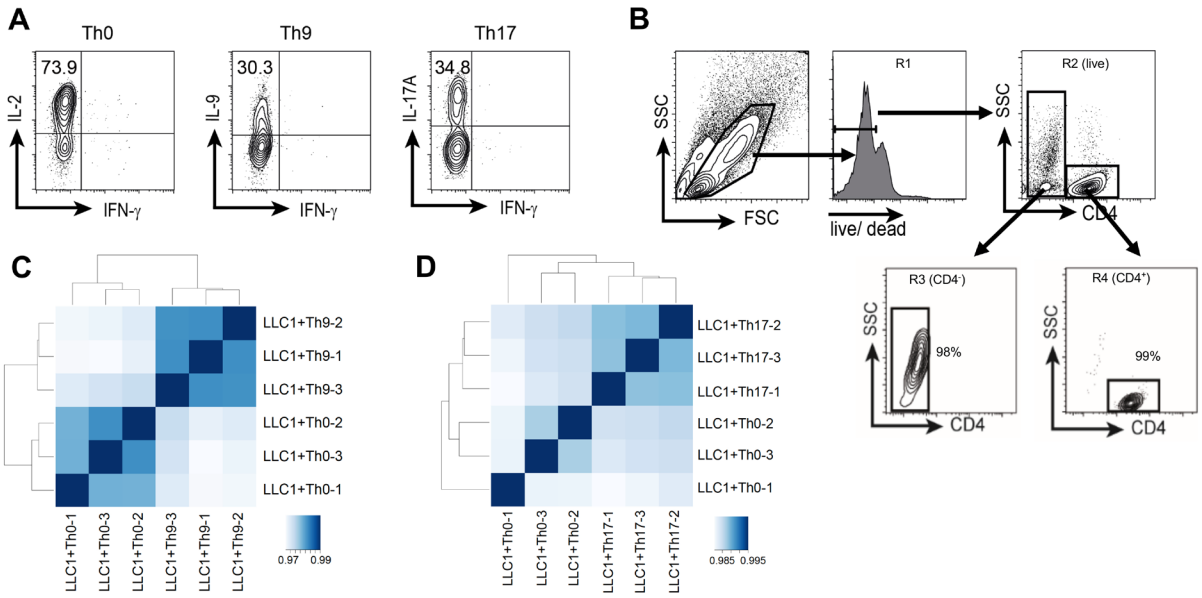
A549 cells (n = 3 donors). (**B**) Quantification of protein expression of EMT markers E-cadherin and Vimentin after 48h stimulation of PTCs (n = 3 donors). In A and B *P < 0.05, **P < 0.01, ***P < 0.001 compared to A549 or PTCs using one-way ANOVA Dunnett's test.



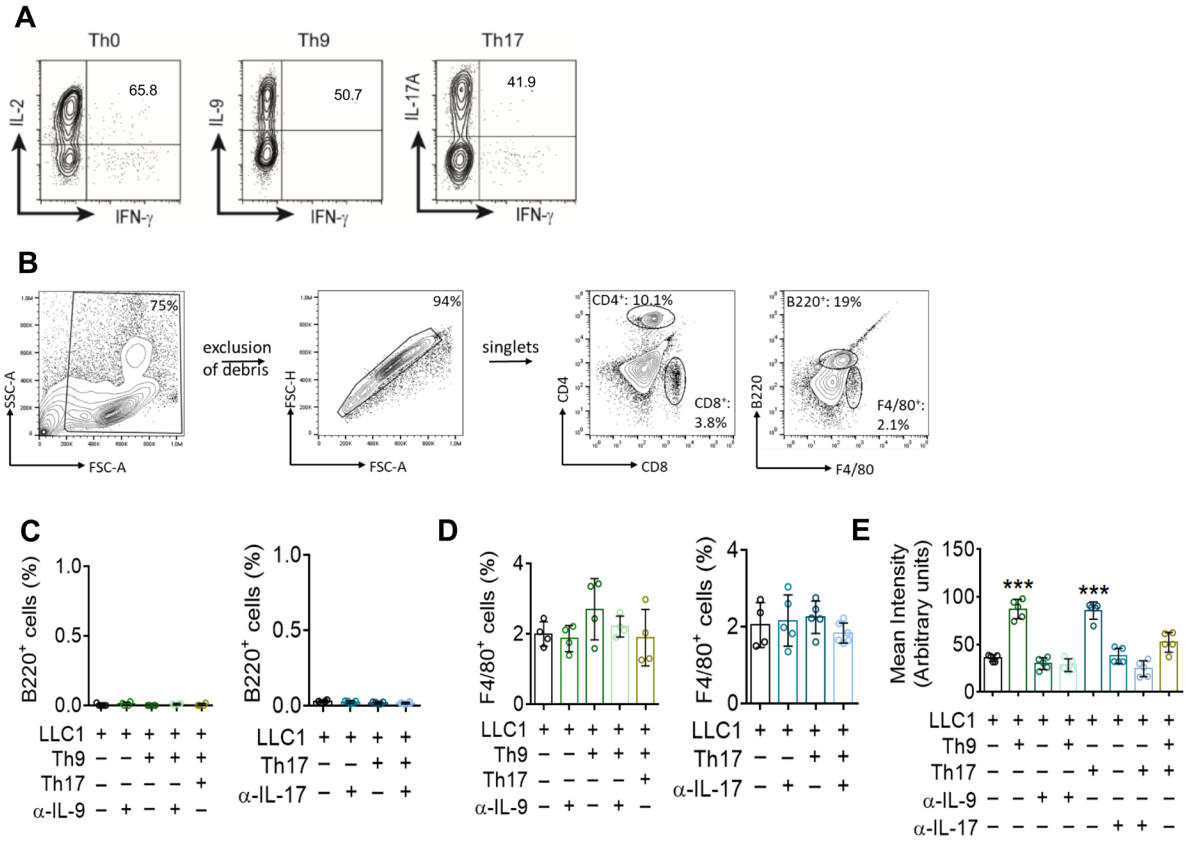
Supplementary Figure 4. Gating strategy for human memory CD4$^+$ T cell subsets: Tumor was dissociated to obtain a single cell suspension, isolated immune cells were stained and acquired. Acquired cells were first gated for live/ dead staining (Zombie-NIR), then gated for exclusion of debris (FSC-A vs. SSC-A). Memory CD4$^+$ cells were identified by gating of CD4$^+$CD45RA$^-$ cells, in which IL-9 and IL-17 were then analyzed.



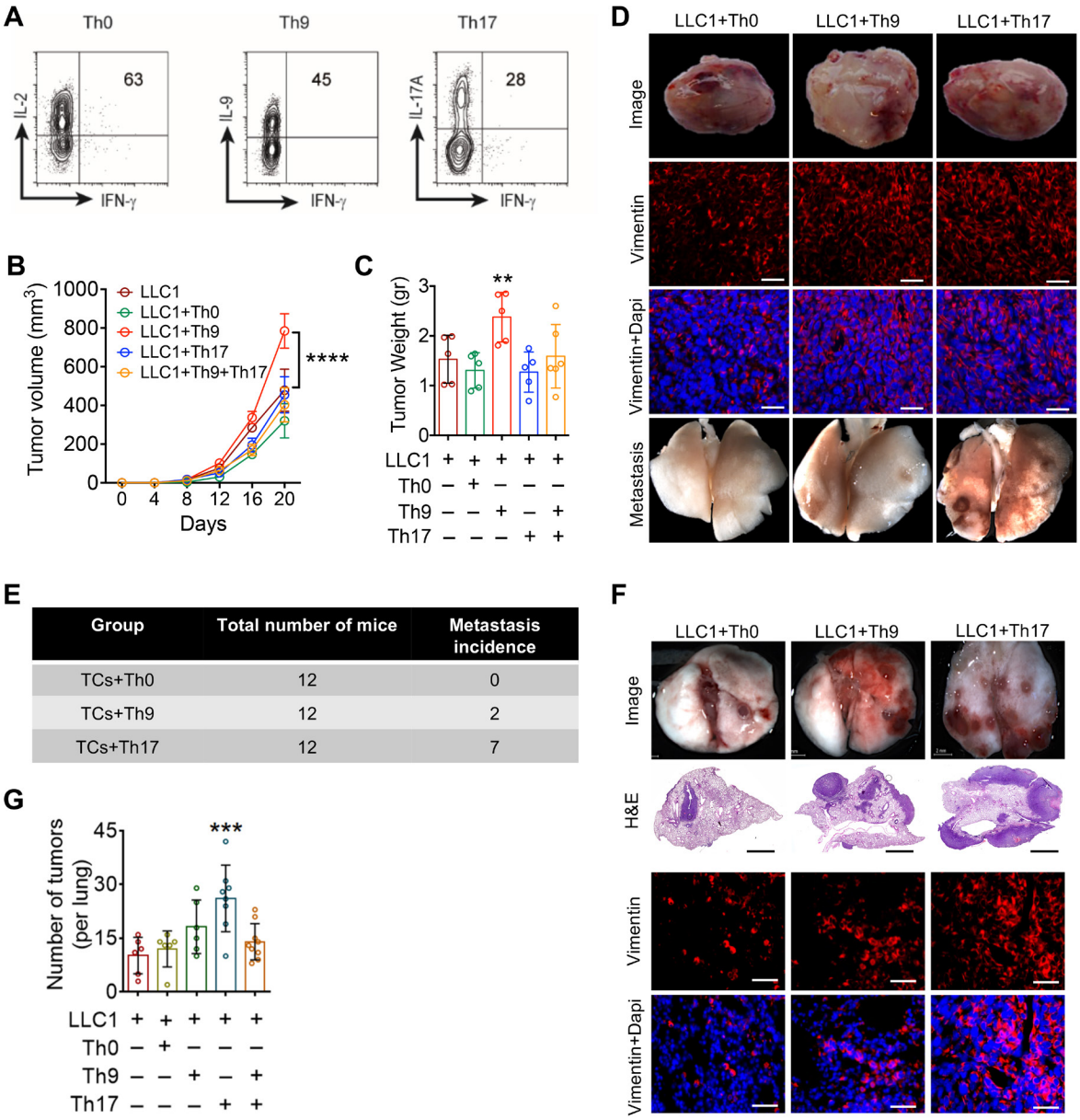
Supplementary Figure 5. CD4$^+$ T-cell subtype specific CM deregulates protein expression of EMT markers: (**A**) Quantification of protein expression of EMT markers N-cadherin, Fibronectin, Vimentin, ZO2 and Cytokeratin18 after 48h stimulation of LLC1 cells. (**B**) Quantification of protein expression of EMT markers N-cadherin, Fibronectin, Vimentin, ZO2 and Cytokeratin18 after 48h stimulation of LLC1 with co-culture CM. (n = 3 donors) in **A** and **B** *P < 0.05, **P < 0.01, ***P < 0.001 compared to LLC1 using one-way ANOVA Dunnett's test.



Supplementary Figure 6. (**A**) Naive $CD44^{-}CD62L^{+}CD4^{+}$ T cells were isolated from C57BL/6 mice and then treated with specific cytokines for 2 – 3 days in order to differentiate into Th9 (TGF-β and IL-4), Th17 (TGF-β and IL-6) or Th0 (without cytokines) cells. Differentiation was confirmed by performing analysis for cell-specific cytokines like IL-9, IL-17, IL-2 and IFN-γ by flow cytometry. (**B**) After 24h of co-culture, cells were sorted to remove all $CD4^{+}$ T lymphocytes and RNA sequencing was performed on remaining LLC1 cells. (**C and D**) Spearman correlation of RNA-seq data displaying the similarity of replicates and conditions per experiment.

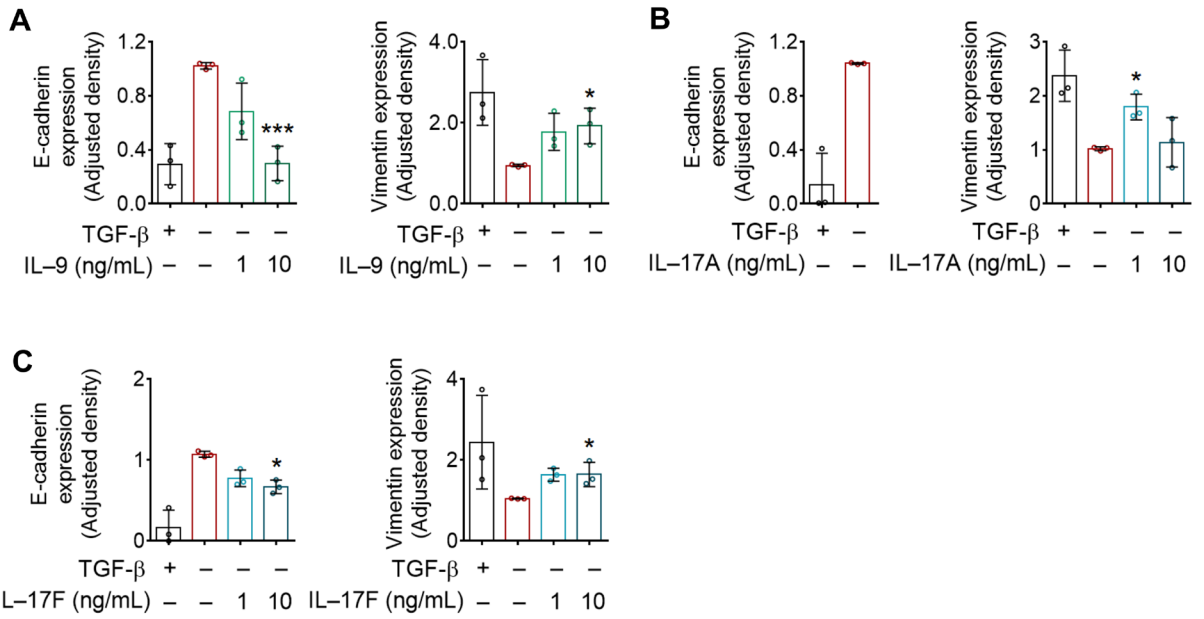


Supplementary Figure 7. Flow cytometry analysis of cytokine production by artificially generated Th0, Th9 and Th17 cells: (**A**) Naive $CD44^{-}CD62L^{+}CD4^{+}$ T cells were isolated from C57BL/6 mice and treated with specific cytokines for 2 - 3 days to differentiate into Th9 (TGF-β and IL-4), Th17 (TGF-β and IL-6) or Th0 (without skewing cytokines) cells. Differentiation was confirmed by performing analysis for cell-specific cytokines IL-2, IL-9, IL-17, and IFN-γ production by flow cytometry. (**B**) Flow cytometry gating strategy for analysis of the frequencies of $CD4^{+}$, $CD8^{+}$, $B220^{+}$ and $F4/80^{+}$ cells. Acquired cells were first gated for exclusion of debris (FSC-A vs. SSC-A), then on single cells (FSC-H vs. FSC-A. $CD4^{+}$, $CD8^{+}$, $B220^{+}$ and $F4/80^{+}$ cells were identified based on the expression of CD4, CD8, B220 and F4/80 markers. (**C and D**) FACS analysis of B cells ($B220^{+}$) and macrophages ($F40/80^{+}$) in lungs from treated mice after co-injection (n=4) (**E**) Mean intensity measurement for Vimentin from immunofluorescence staining of tumor sections (n=5). ***$P < 0.001$ compared to LLC1 control group using one-way ANOVA Dunnett's test.

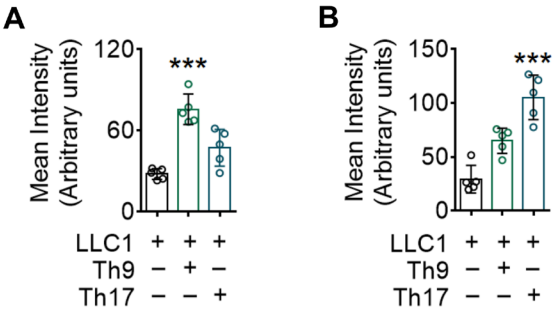


Supplementary Figure 8. Co-injection of LLC1 cells with Th9 and Th17 cells in WT mice leads to increased primary tumor growth and metastasis in vivo: (**A**) Naive $CD44^{-}CD62L^{+}CD4^{+}$ T cells were isolated from C57BL/6 mice and then treated with specific cytokines for 2 – 3 days in order to differentiate into Th9 (TGF-β and IL-4), Th17 (TGF-β and IL-6) or Th0 (without cytokines) cells. Differentiation was confirmed by performing analysis for IL-9, IL-17, IL-2 and IFN-γ production by flow cytometry. Afterwards, Th0, Th9 or Th17 cells were co-injected with LLC1 cells into C57BL/6 mice (**B–E**) subcutaneously or (**F–G**) intravenously. (**B and C**) Tumor size, tumor weight of extracted tumors of co-injected groups (n=5). (**D-E**) Representative images of tumors, immunofluorescence staining for Vimentin (red)

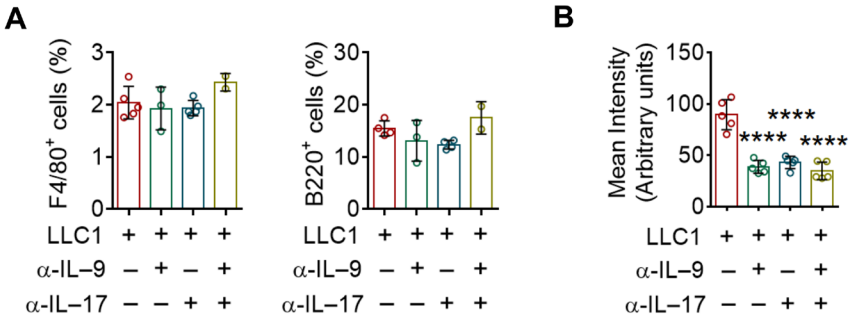
and DAPI (blue) in sections, extracted lungs and table depicting metastasis incidence. (**F**) Representative pictures of extracted lungs, hematoxylin & eosin (H&E) stained sections and immunofluorescence staining for Vimentin (red) and DAPI (blue) in tumor sections after intravenous co-injection of Th0, Th9 or Th17 cells with LLC1 cells. In **D** and **F** scale bars for immunofluorescence: 50µm. In **F** scale bars for H&E staining: 1.5mm. (**G**) Quantification of macroscopic lung tumor nodules (n = 6). **P < 0.01, ***P < 0.001 in **B**, **C** and **G** compared to LLC1 co-injection group using one-way ANOVA Dunnett's test.



Supplementary Figure 9. Stimulation with IL-9 and IL-17 results in decreased E-cadherin and increased Vimentin protein levels in A549 cells: (**A-C**) Quantification of protein expression of E-cadherin and Vimentin in A549 cells after 48h stimulation with IL-9 (**A**), IL-17A (**B**) and IL-17F (**C**). (n = 3 experimental replicates) in **A-C** **P < 0.01, ***P < 0.001 compared to unstimulated A549 using one-way ANOVA Dunnett's test.



Supplementary Figure 10. EMT marker Vimentin is increased in the tumor stroma of co-injection mice with impaired IL-9 and IL-17 signaling: (**A**) Mean intensity measurement for Vimentin from immunofluorescence staining of tumor sections of *Il17ra*$^{-/-}$ mice. (**B**) Mean intensity measurement for Vimentin from immunofluorescence staining of tumor sections from *Il9r*$^{-/-}$ mice. (n=5) ***P < 0.001 compared to LLC1 control group using one-way ANOVA Dunnett's test.



Supplementary Figure 11. Macrophages and B cells display no change after antibody treatment: (**A**) FACS analysis of macrophages (F40/80^{+}) and B cells (B220^{+}) in lungs from WT treated mice (n=2-4) (**B**) Mean intensity measurement for Vimentin from immunofluorescence staining of tumor sections of WT mice. (n=5) ****P < 0.0001 compared to control using one-way ANOVA Dunnett's test.