Supplementary Materials and Methods

Lung immunofluorescence.

After sacrifice, healthy and tumor-bearing KP-XCR1^{Venus} lungs were harvested after PBS intracardiac perfusion. Then, PBS 1% paraformaldehyde (PFA) was intratracheally perfused to fix the lung tissue. Left lobes was then placed in PBS 4% PFA ON, and subsequently passed in PBS 15-30% sucrose. Finally, lungs were embedded in Killic - O.C.T. (Bio-Optica, Cat# 05-9801) and 5 µm sections were obtained in a cryostat. Lung sections were defrosted and immediately fixed with PBS 4% PFA for 10 minutes. Afterwards, cryosections were washed twice with Washing buffer (PBS, 1% BSA, 0.1% Triton X-100) for 5 minutes at RT, and blocked with Blocking buffer (Washing buffer, 5% mouse serum) for 40 minutes at RT. Then, lungs were incubated ON at 4°C with a rat anti-mouse CD8 antibody (Invitrogen, Cat# 14-0808-82) in Blocking buffer. After ON incubation, sections were washed twice with Washing buffer for 5 minutes at RT and incubated for 4 hours at RT with a goat anti-rat-AF647 secondary antibody (Invitrogen, Cat# A-21247). After secondary antibody, sections were washed with PBS for 5 minutes at RT, and nuclei were stained with Hoechst 33342 (Invitrogen, Cat# H3570) in PBS for 20 minutes at RT. Lastly, lung sections were washed twice with PBS for 2 minutes at RT and mounted with Mowiol 40-88 (Sigma-Aldrich, Cat# 324590). Confocal microscopy images were acquired using a Zeiss LSM 880 instrument, and subsequently analyzed in ImageJ 1.54f software.

cDC1 isolation from lung tissues.

Healthy and tumor-bearing lungs were harvested after PBS intracardiac perfusion. Tissues were mechanically cut and digested in complete media containing Collagenase type 2 (265 U/mL; Worthington, Cat# LS004176) and DNase I (250 U/mL; Roche, Cat# 11284932001) at 37 °C for 30 minutes. Digestion was then stopped by adding EDTA (10 mM; Invitrogen, Cat# 15575-038), tissue pieces were passed through a 18G needle and then filtered using 100 µm cell strainers (Starlab, Cat# CC8111-0102) to obtain single-cell suspensions. Red blood cells were eliminated with ACK Lysing buffer (Gibco, Cat# A10492-01). Afterwards, CD11c^{+} cells were isolated using magnetic CD11c-microbeads (Miltenyi Biotec, Cat# 130-125-835) following manufacturer's instructions. Isolated cells were then stained with anti-mouse antibodies in FACS buffer (PBS, 1% BSA) for 30 minutes at 4°C: CD11c-APC (Cat# 117310), XCR1-BV650 (Cat# 148220), CD172a/Sirpα-PE-Dazzle 594 (Cat# 144015) purchased from BioLegend, and CD170/SiglecF-BB515 (Cat# 564514) and MHC-II (I-A/I-E)-BV711 (Cat# 563414) purchased from BD

Biosciences. After washing, stained cells were resuspended in PBS 2% FBS and cDC1 were purified in a FACS Aria II Cell Sorter (BD).

OT-I isolation.

Naïve OT-I or OT-I^{TdTomato} cells were isolated from lymph nodes using magnetic CD8-microbeads (Miltenyi Biotec, Cat# 130-116-478) following the manufacturer's instructions. Effector OT-I were obtained by co-culturing naïve OT-I for two days with bone marrow-derived DCs (BMDCs), which were previously pulsed for 3 hours with 30 nM SIINFEKL and 1 µg/ml poly(I:C) (Invivogen, Cat# tlrl-pic). BMDCs were obtained by differentiating freshly isolated bone marrow cells in complete IMDM media 30% GM-CSF for 7 days.

OT-I proliferation assays.

Sorted lung cDC1 were plated in tissue-treated 96-well U-bottom plates (5,000 cDC1 per well) in complete IMDM 10% FBS for 3 hours at 37°C, in the presence of SIINFEKL at different concentrations (0; 0.3 and 1 nM) and 1 µg/ml poly(I:C). After cDC1 were washed, 50,000 naïve CellTrace Violet (CTV)-labelled OT-I cells (5 µM CTV; Invitrogen, Cat# C34571) were added to each well in complete RPMI media 10% FBS and kept at 37°C for 72 hours (1:10, cDC1:OT-I ratio). After 48 hours of co-culture, aliquots of conditioned media were collected for measuring IL-2 and IFNγ by ELISA (BioLegend, Cat# 431001 and 430801). Cells were washed and stained with anti-mouse CD3ε-FITC (Cat# 100305) and CD8a-APC (Cat# 100712), purchased from BioLegend, in FACS buffer for 30 minutes at 4°C. Afterwards, cells were washed with PBS and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, Cat# L34966) according to manufacturer's instructions. Finally, cells were fixed in FACS-Fix buffer (PBS, 1% PFA), and CTV dilution was analyzed in a FACS Celesta Cell Analyzer (BD). Data generated were further analyzed in FlowJo software (10.8.1 version, BD).

Flow cytometry analysis of lungs and lymph nodes.

Lungs from tumor-bearing mice (autochthonous and transplantable models) were harvested and processed to obtain single-cell suspensions as previously described. Mediastinal lymph nodes (medLNs) were obtained previous to PBS intracardiac perfusion, and smashed through 100 µm cell strainers using a syringe plunger to obtain single-cell suspensions. Fc receptors were blocked with anti-mouse CD16/32 antibodies (BioLegend, Cat# 101320) in FACS buffer for 10 minutes at 4°C. Single-cell suspensions were then washed and stained with anti-mouse antibodies in FACS buffer for 30 minutes at 4°C, as follows: lungs: CD45-APC/Fire750 (Cat# 103154), lineage-FITC

[CD19-FITC (Cat# 115505), CD3ε-FITC, CD45R/B220-FITC (Cat# 103206), NK1.1-FITC (Cat# 108705)], CD11c-BV785 (Cat# 117335), MHC-II (I-A/I-E)-AF700 (Cat# 107622), XCR1-BV650, CD366/Tim3-PEDazzle594 (Cat#119747), CD223/Lag3-BV650 (Cat#125227), CD279/PD-1-BV421 (Cat#135221) and CD172a/Sirpα-PE-Dazzle 594 purchased from BioLegend, CD11b-BV421 (BD Biosciences, Cat# 562605), CD170/SiglecF-PerCP-Cyanine5.5 (BD Pharmingen, Cat# 565526), and CD274/PD-L1-PerCP-eFluor710 (eBioscience, Cat#46-5982-80). medLNs: lineage-FITC, CD11b-BV421, CD11c-BV785, MHC-II (I-A/I-E)-AF700, and XCR1-BV650. Anti-mouse antibodies for adhesion molecules were used, as follows: CD273/PD-L2-PE (Cat# 107205), CD44-PE (Cat# 103007), and MHC-I (H-2Kb)-PE (Cat# 116507) purchased from BioLegend, and ALCAM-APC (Invitrogen, Cat# 17-1661-80). After staining, cells were washed with PBS, stained with LIVE/DEAD kit as previously described and fixed in FACS-Fix buffer. Cells were analyzed in a FACS Celesta Cell Analyzer (BD). To quantify cDC1 absolute numbers, CountBrigh Absolute Counting Beads (Invitrogen, Cat# C36950) were added to samples before being analyzed. Data generated were further analyzed in FlowJo software (10.8.1 version, BD).

Detection of specific OVA-MHC-I complexes on cDC1

To detect specific MHC class-I OVA complexes on cDC1, total $CD11c^+$ cells were isolated from early or late tumor tissues using microbeads (Miltenyi, Cat#130-125-835), loaded for 90 minutes with 270 nM SIINFEKL plus 1 µg/mL Poly (I:C) and incubated for 1 hr at 37 °C with PE-coupled 25-D1.16 mAb (Invitrogen, Cat# 12-5743-81). One sample not loaded with peptide was used as control. Cells were washed and stained with extracellular antibodies to identify cDC1 and analyzed by flow cytometry.

ALCAM staining in cDC1-CD8 T-cell conjugates.

Ex vivo cDC1-CD8 T-cell conjugates were prepared as previously described, using sorted early (d8) $cDC1^{Venus}$ and naïve $OT\text{-}I^{TdTomato}$ cells. After incubation, cells were washed once with cold PBS and stained in coverslips with anti-mouse ALCAM-APC antibody in FACS buffer for 30 minutes at 4°C. Afterwards, cells were fixed with PBS 1% PFA for 15 minutes at RT, followed by permeabilization and blocking in Blocking buffer (PBS, 0.2% BSA, 0.05% saponin, 5% horse serum) for 40 minutes at RT. Finally, nuclei were stained with Hoechst 33342 in PBS for 20 minutes at RT. Lastly, coverslips were washed twice with PBS for 2 minutes at RT and mounted with Mowiol 40-88. Confocal microscopy images were acquired and analyzed as previously described.

The average intensity map distribution for ALCAM was obtained using a custom-written macro in ImageJ 1.54f. Single plane images of cDC1^{Venus} either alone or conjugating with OT-I^{TdTomato} were oriented in the same direction (cDC1^{Venus} on the left and OT-I^{TdTomato} on the right) and size normalized. Representative images show the average normalized distribution of ALCAM obtained by integrating all the normalized cells for each condition. In parallel, ALCAM integrated density at the synapse or the opposed side in cDC1^{Venus} was calculated and normalized by the total ALCAM density of the cell.

Coating of beads with ALCAM ligand.

Polybead amino 6 µm-diameter microspheres (Polysciences, Cat# 19118) were prepared to covalently couple the ALCAM ligand CD6. Briefly, beads were washed thrice with PBS, centrifuging at 5,000 x *g* for 5' at 4°C in each step, and activated with glutaraldehyde 8% in PBS for 3 hours at RT, mixing continuously in an orbital rotor. Beads were then washed thrice with PBS to remove glutaraldehyde, and incubated over-night at 4°C with 10 µg/ml of either recombinant mouse CD6-Fc chimera, rCD6(Fc), or the correspondent recombinant human IgG1 Fc molecule (rFc) as control in PBS, in an orbital rotor.

After washing with PBS to remove any traces of unbound protein, beads were blocked with PBS 1% BSA for 30' at RT, with continuous mixing in an orbital rotor. Lastly, after washing with PBS beads were resuspended in PBS 1% BSA (50,000 beads/µl) and kept at 4°C until use.

ALCAM-CD6 interference experiments.

For ALCAM interference experiments in *ex vivo* cDC1-CD8 T-cell conjugates, rCD6(Fc) was added to cDC1 while attaching to fibronectin-coated coverslips and maintained during the cDC1-OT-I interacting phase at a final concentration of 10 µg/ml. As a control, rFc was used at the same concentration.

For ALCAM interference in T-cell activation experiments, sorted lung cDC1 were plated in tissue-treated 96-well U-bottom plates (5,000 cDC1 per well) in complete IMDM 10% FBS for 3 hours at 37°C, in the presence of 1 nM SIINFEKL and 1 µg/ml poly(I:C). After, cDC1 were washed and 50,000 naïve OT-I cells were added to each well in complete RPMI media 10% FBS and kept at 37°C (1:10, cDC1:OT-I ratio). rCD6(Fc) or rFc was added to the co-culture at a final concentration of 2 µg/ml. After 48 hours of co-culture, aliquots of conditioned media were collected for measuring IL-2 and IFNγ by ELISA.

Mouse datasets analysis.

Microarray data from purified control and KP-lung cDC1 was previously generated in our laboratory[3], is publicly available in Gene Expression Omnibus (GEO) under accession code GSE119574. scRNA-seq data from control and KP lungs were obtained from GEO under accession code GSE131957[2]. Briefly, the R object file containing expression values and cell annotations was downloaded from the GitHub repository associated to the publication (https://github.com/effiken/Maier_et_al_nature_2020). Cell adhesion molecules' genes were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database, especially focalizing in those adhesion molecules involved in immune system cell-to-cell interactions (https://www.genome.jp/pathway/hsa04514). Genes were confronted with our microarray data to find those differentially expressed (DEGs) between control and KP cDC1 (p value <0.05). Afterwards, DEGs were validated in the scRNA-seq dataset.

Alcam expression values in steady-state mouse dendritic cells from lung, spleen and liver, as well as lung macrophages were retrieved from the Immunological Genome Project (ImmGen; https://www.immgen.org/) Microarray V1 Dataset. Expression values were $\log_2$ transformed and z-score normalized for visualization.

Human datasets and survival analysis.

ALCAM expression in LUAD patients was analyzed in two curated microarray experiments from the GEO Dataset Browser (www.ncbi.nlm.nih.gov/sites/GDSbrowser/), in which matched samples from lung tumor and noninvolved areas from the same patient were present: GSE10072 (33 patients with matched samples) and GSE19804 (60 patients with matched samples). *ALCAM* expression values were obtained through the GEO2R platform, then data were $\log_2$ transformed and z-score normalized.

To analyze the predictive value of *ALCAM* expression in the lung with overall survival in LUAD patients, we performed a survival analysis through the online platform KM plotter (www.kmplot.com/) for lung cancer[27], with the following settings: Affy id: 201952_at (ALCAM JetSet optimal probe); split patients by: trichotomization (lower tercile [T1] *vs* upper tercile [T3]); Overall Survival (OS); Follow up threshold: all; Restrict analysis to subtypes: Histology: adenocarcinoma; Cox regression: univariate; Array quality control: exclude biased arrays. These settings resulted in a survival analysis between the *ALCAM*-low group, composed of 383 patients, and the *ALCAM*-high group, composed of 395 patients. The resulting hazard ratio (HR), 95% confidence interval, and the Mantel-Cox (logrank) test were provided by the KM plotter platform.

Supplementary references

27. Győrffy B. Transcriptome-level discovery of survival-associated biomarkers and therapy targets in non-small-cell lung cancer. Br. J. Pharmacol. 2024;181(3):362–374. doi:10.1111/bph.16257