Supplementary Infomation for

Comprehensive profiling of smoke-induced T cells reveals clonal γδT17 cells as a hallmark of COPD

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The PDF file includes:

Supplementary methods

Cell preparation

Mice were deeply anesthetized. Bronchoalveolar lavage fluid (BALF) was acquired by inflating lungs with 1 mL of complete RPMI 1640 medium via tracheal cannulation, followed by three lavages. Cells in the BALF were collected by centrifugation. Right ventricular perfusion was performed with 10 mL of ice-cold PBS and dissected the lungs. The lungs were washed in PBS and minced into small pieces with scissors. The lung tissue was digested for 30 min at 37 °C in HBSS containing 2% heat-inactivated FBS, 100×Pen Strep, 2mM L-glutamine, 25 mM HEPES, 1mg/mL Collagenase D and 0.1mg/mL DNase I. The spleen was dissected, and splenocytes were isolated using mechanical dissociation protocol. Cell suspension was filtered through a 70 mm strainer. Red blood cells were lysed as required.

γδT17 cell expansion culture and adoptive transfers

Pooled spleen cells from IL17-GFP mice were cultured at 1×10^6 cells per mL in RPMI 1640 containing 10% heat-inactivated FBS, 100 × Pen Strep, 2mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 55 nM 2-Mercaptoethanol and 1× MEM Non-Essential Amino Acids Solution. The culture conditions included 5 ng/mL recombinant rIL-23, 5 ng/mL rIL-1β and 10 mg/mL anti-mouse IFNγ. Cells were plated in 48-well round-bottom plates coated with anti-mouse TCR γ/δ at a concentration of 1 μg/mL and incubated for 3 days. After the initial incubation, cells were washed and re-seeded on fresh plates at 1×10^6 cells per mL under the same conditions but without TCR-γδ stimulation and cultured for additional 3 days. The cells were then washed and re-seeded in medium containing 20 ng/mL rIL-7 and 10 μg/mL a-IFN-γ for a further 3 days. Antibodies and reagents used in this protocol were shown in Supplementary Table 1. At day 9 of culture, γδT17 cells were harvested and purified by FCAS. To purify in vitro expanded γδT17 cells by removing Th17 cells, we used surface staining combined flow cytometric sorting with the $CD4^-$APC-Cy7 antibody listed in Supplementary Table 1. Gating strategies are detailed in Supplementary Fig. 10. The collected cells were washed twice and resuspended in PBS, and 2×10^6 cells were intravenously injected into *Tcrd*$^{-/-}$ mice. A solvent control group (PBS injection only) was also established. After confirming no procedural errors within 24 hours, the mice were transferred to a smoke exposure box for exposure.

BD Rhapsody sequencing library preparation and sequencing.

Whole transcriptome analysis (WTA) and targeted transcriptome analysis (TTA) combined with TCR-seq of FCAS enriched live T cells (airway, air-par, parenchyma, spleen) were performed using the BD Rhapsody Single-Cell Analysis System (BD, Biosciences). Cells were pooled from four mice per sample, with 3 parallel replicates (n = 3). Next, 12 barcoded samples were pooled for a total of 40,000 or 60,000 cells, and centrifuged at 400g for 5 min. The pellet was resuspended in 650 μL BD Sample Buffer from the BD Rhapsody Cartridge Reagent Kit (633731, BD Biosciences). Single cells were isolated using the BD Rhapsody Express Single-Cell Analysis System with BD Rhapsody Cartridge Kit (633733, BD Biosciences)with, following to the manufacturer's recommendations. cDNA libraries were prepared using the BD Rhapsody cDNA Kit (633773, BD Biosciences), BD Rhapsody WTA Amplification Kit (633801, BD Biosciences), and BD Rhapsody Immune Response Panel Mm (633753, BD Biosciences) following the BD Rhapsody System manufacturer's Protocol. The final libraries were quantified with a Qubit Fluorometer using the Qubit dsDNA HS Assay Kits (Q32851, Thermo Fisher Scientific). Information about the critical commercial assays and products used for sequencing library preparation is shown in Supplementary Table 1. Single cell sequencing data were collected by Illumina Novaseq 6000.

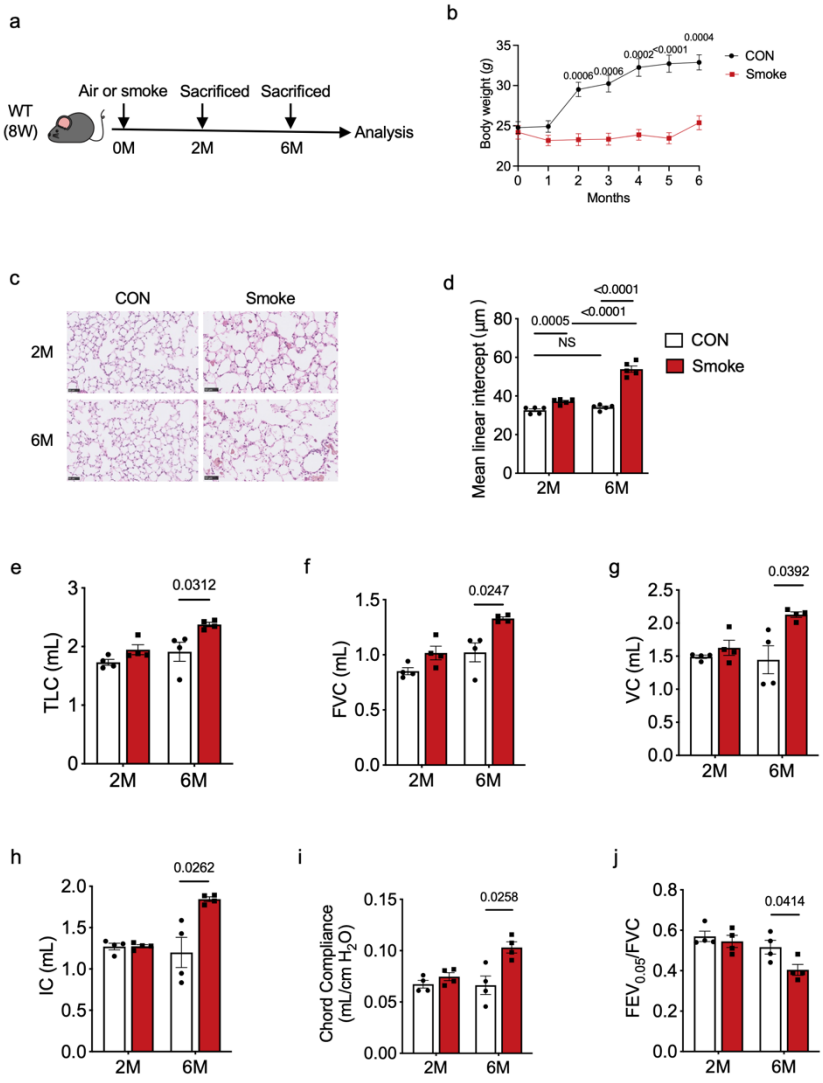
BD Rhapsody sequencing preprocessing

The sequencing data were processed using the BD Rhapsody data processing pipeline version 1.9.1 specific for TTA and WTA, respectively. In the pipeline, the data was firstly filtered by read quality, with the following criteria: 1) the length of R1 no less than 60bp and R2 length no less than 42bp; 2) The mean base quality score must be larger than 20 for both R1 and R2; 3) the highest SNF have to fulfill R1<0.55 and R2<0.8. Subsequently, R1 reads were annotated to a pre-designed cell label pool, while R2 reads were trimmed the head 5bp and aligned to the BD Rhapsody Immune Response Panel reference with bowtie2 in the TTA pipeline. Only the R2 reads with no less than 37bp in length after alignment were considered for further processing. For WTA data, the R2 reads were aligned to GRCm38 reference by STAR and only uniquely mapping reads were kept for further processing. The raw read count matrix was constructed by collapsing reads with same cell label, UMI and gene. BD Rhapsody developed RSEC method was applied to further adjust gene expression in both WTA and TTA pipelines. For TTA pipeline, an additional BDEC

method followed the RSEC step to further correct UMI errors derived from library preparation and sequencing base deletions. A final cell label filtering step generated the single-cell gene expression matrix. TCR sequencing data processing followed a similar approach to TTA, with the VDJ version option set to ‘mouseTCR’.

Processing data with Seurat Package

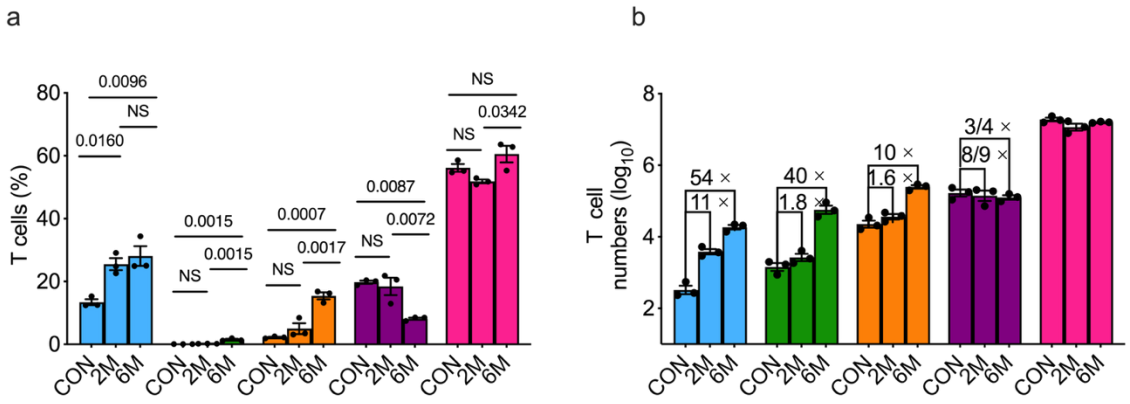
The single-cell gene expression matrix was further analyzed using the ‘Seurat 4.0.1’ R package. The Reads Per Cell data matrix produced by BD Rhapsody pipeline was loaded, and the CreateSeuratObject was used to create the object data for further analysis. Files named ‘Sample_tag_Calls’ from the BD pipeline results were also loaded to assign sample tags to individual cells. The designation of sample tags can be found in Supplementary Table 2. The BD Rhapsody pipeline integrated sample tag DNA sequences into the reference prior to sequence mapping. And a valid singlet cell was defined as one with at least 75% of its sample tag DNA mapping correctly to a sample. According to the sample tag design, tissue information and treatment time for each single cell were gathered and integrated into the Seurat object data.



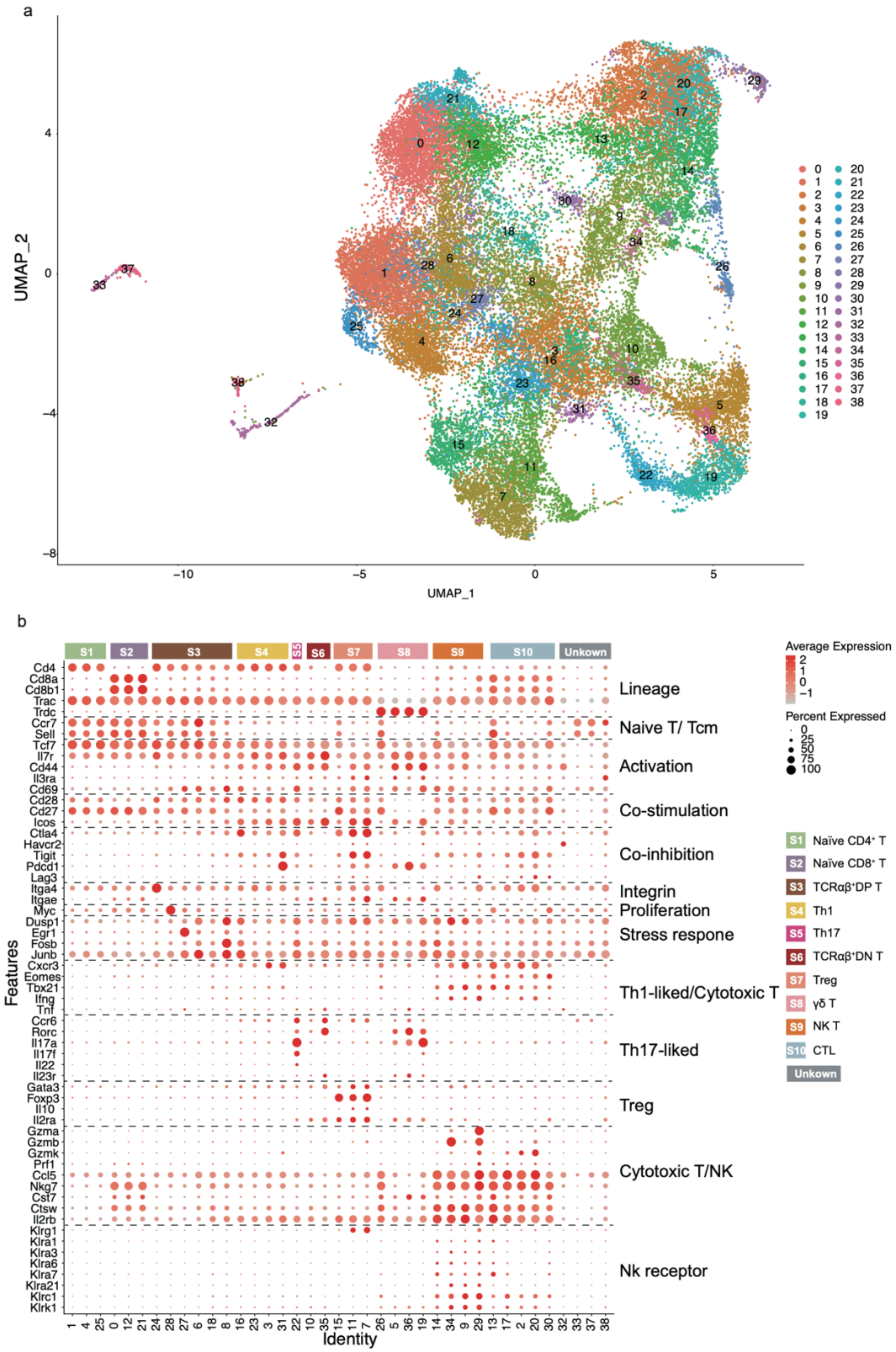
Supplementary Fig. 1. Lung histology and lung function tests revealed that tobacco smoke exposure leads to emphysema-like and airflow-limited phenotype in mice.

a, b C57BL/6J wild-type (WT) mice were randomly divided into two groups: the air control group (CON) and the tobacco smoke exposure group (Smoke). Mice were placed in a fume box and exposed to tobacco smoke for 2 or 6 months, respectively, with body weights continuously recorded during the exposure (n = 5 mice/group/time piont). **c** Representative images of hematoxylin and eosin-stained paraffin sections (40 ×, scale bar = 50 μm) of the WT groups show that after 2 months (2M) or 6 months of tobacco smoke exposure (6M), the Smoke group exhibited marked airway inflammation and fused alveolar damage compared to the CON group. The experiment was repeated independently at least three times with similar results. **d** The mean

alveolar intervals (MLI) were significantly increased in the Smoke group (n = 5 mice/group/time piont). **e-j** Pulmonary function tests showed significant differences in (E) total lung capacity (TLC), (F) forced vital capacity (FVC), (G) vital capacity (VC), (H) inspiratory capacity (IC), (I) chord compliance between 0 and 10 cmH_2O (Cchord), and (J) FEV_{50}/FVC under both air control and tobacco smoke exposure conditions (n = 4 mice/group/time piont). Data are mean ± SEM. Two-sided unpaired Student's t-test was used to analyze differences in the mean values between groups, while the Mann-Whitney test was applied to compare the central tendencies (mean or median) between the two groups. NS, not significant.

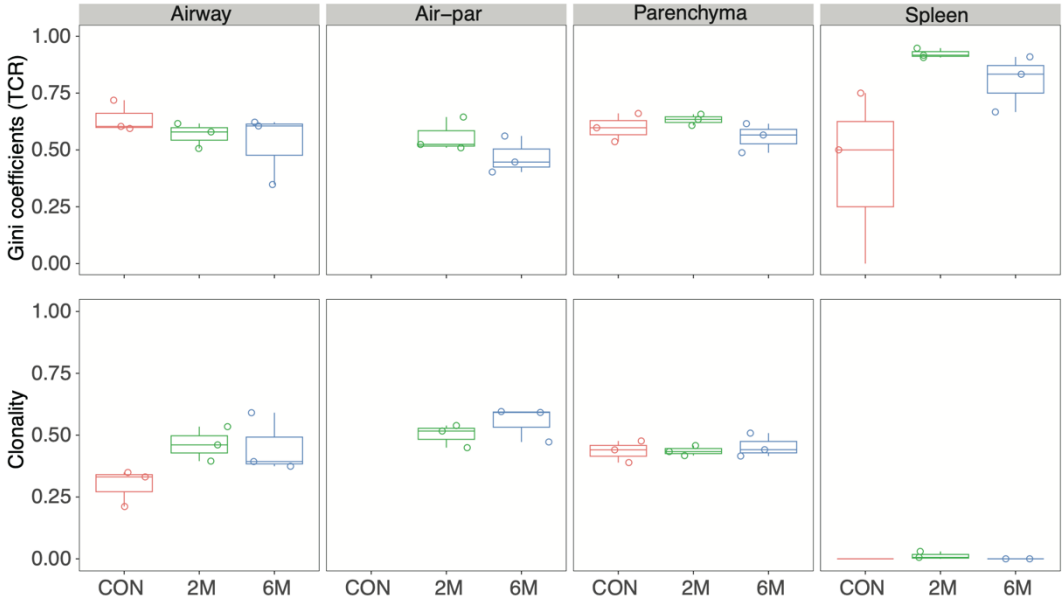


Supplementary Fig. 2 T cells are recruited to the airway after tobacco smoke exposure. Mice were sacrificed at 0 months (n = 3), 2 months (n = 3), and 6months (n = 3) after tobacco smoke exposure. T cells from the airway, the area between airway and parenchyma (air-par), parenchyma, vasculature, and spleen were analyzed by fluorescence-activated cell sorting (FACS). To localize T cells in the respiratory tract after tobacco smoke exposure, 0.25 mg of fluorochrome-conjugated CD45 and 0.5 mg of fluorochrome-conjugated CD90.2 antibodies were administered intranasally (i.n.) and intravenously (i.v.). **a, b** The frequency and/or numbers of T cells are shown. Data are mean ± SEM. One-way ANOVA was used to compare differences among multiple groups when data followed a normal distribution, whereas the non-parametric Kruskal–Wallis test was applied for data that did not meet this assumption. NS, not significant, Air: Airways; Air-par: the airway-parenchyma interface; Par: parenchyma; Spl: Spleen.


a
UMAP_2
UMAP_1
b
Features
Identity
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
Unkown
Lineage
Naive T/ Tcm
Activation
Co-stimulation
Co-inhibition
Integrin
Proliferation
Stress respone
Th1-liked/Cytotoxic T
Th17-liked
Treg
Cytotoxic T/NK
Nk receptor
Average Expression
Percent Expressed
Naïve CD4+ T
Naïve CD8+ T
TCRαβ+DP T
Th1
Th17
TCRαβ+DN T
Treg
γδ T
NK T
CTL
Unkown

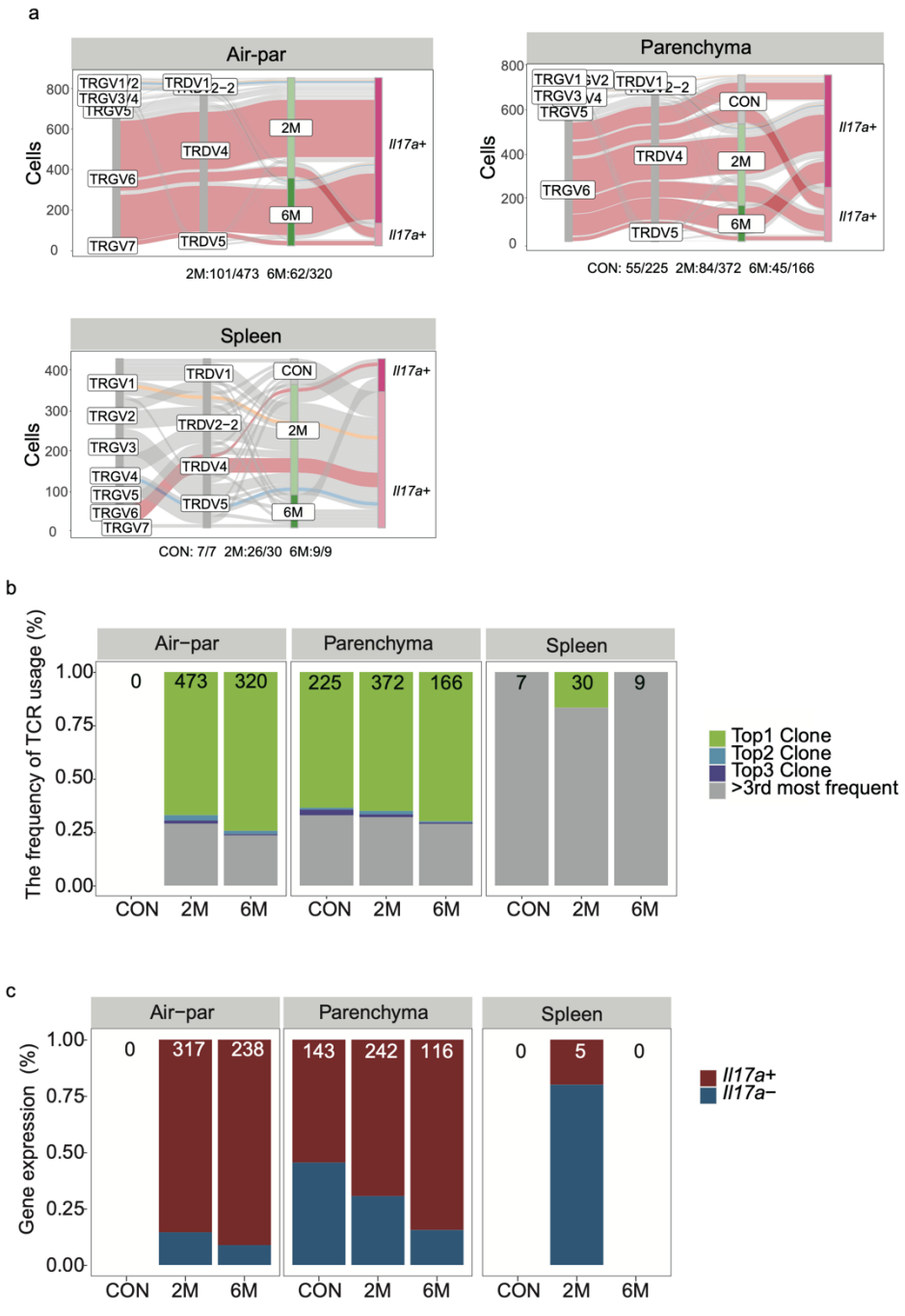
Supplementary Fig. 3. T cells from mice in the air control group and 2mouth or 6 mouth after the tobacco smoke exposure.

a Single-cell transcriptomes of T cells displayed by UMAP. Seurat-based clustering of 40849 cells, colored based on cluster ID. **b** Average expression (color scale) and the percentage of expressing cells (size scale) of selected genes in indicated clusters. The values were row scaled. The marker genes for different cell types were listed on the left.



Supplementary Fig. 4. Distinct clonal expansion patterns within γδ T cells in mice.

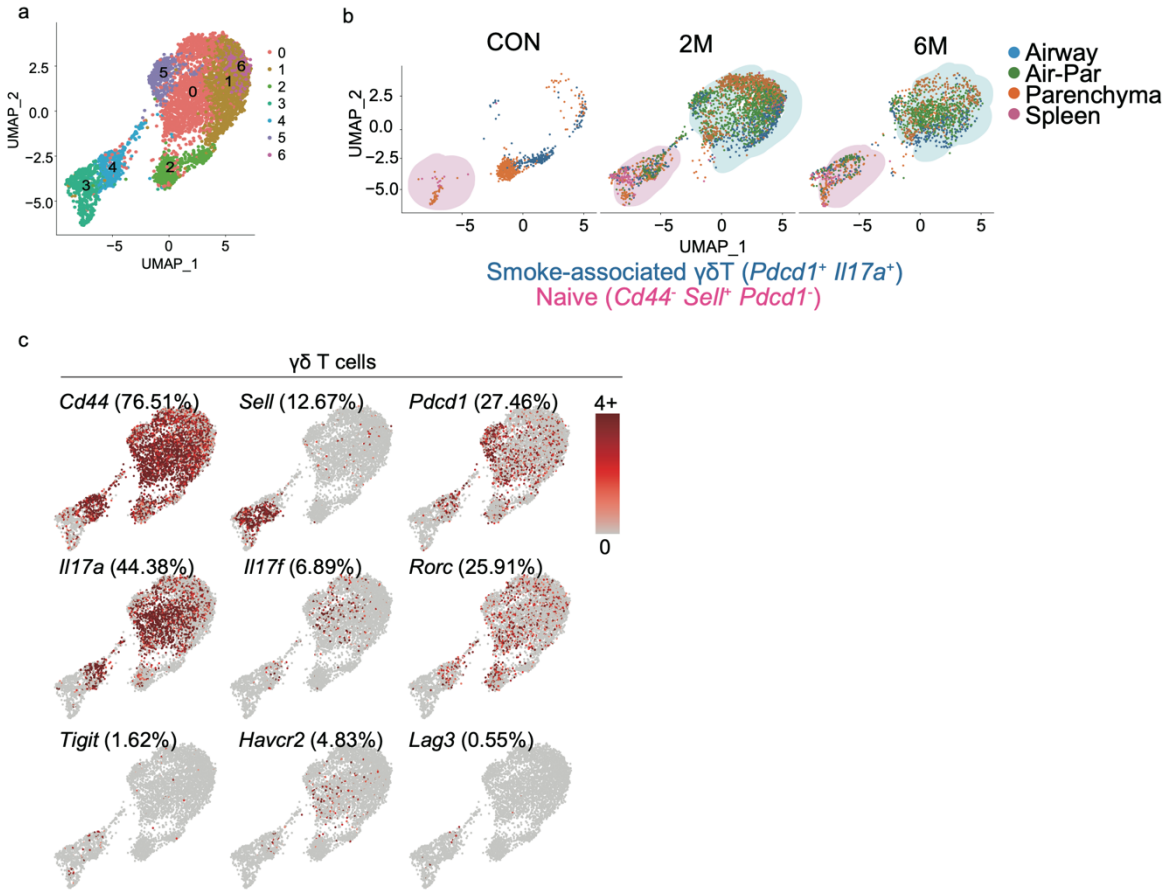
Gini coefficients and clonality show the pattern of TCR clone expansion in γδ T cells from different tissue sites after 2 and 6 months of tobacco smoke exposure. Box plots indicate median (middle line), 25th, 75th percentile (box) and the minimum and maximum values (whiskers) as well as outliers (single points). CON: air control group, n = 3; 2M: 2-month tobacco smoke exposure group, n = 3; 6M: 6-month tobacco smoke exposure group, n = 3.



Supplementary Fig. 5. Effects of smoked on γδ T cell populations in mice.

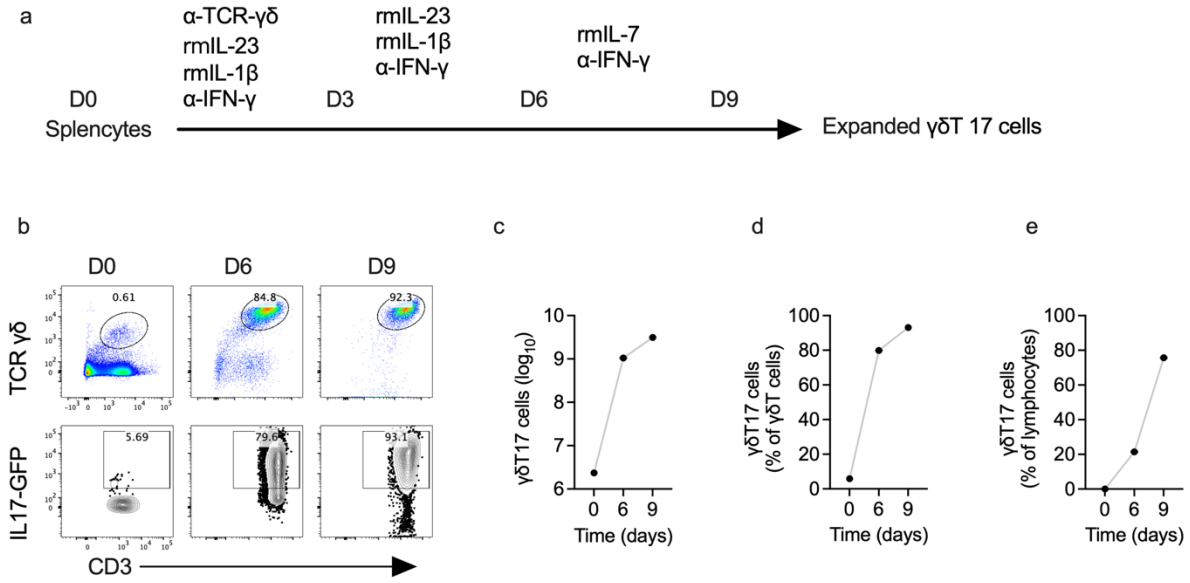
a Parallel plots showing a correlation in the air-par, parenchyma and spleen between the frequency of V gene usage and its pairing with *Il17a* expression in relation to the progression of tobacco smoke exposure. Clones (represented by lines) shared are colored: Top 1 (red), top 2 (blue), top 3 (yellow) and all other clones with low frequency (gray). The number of clones/cells for CON, 2M

and 6M are also shown. **b** Bar graphs illustrating the frequency of TCR clones in γδ T cells in the air-par, parenchyma and spleen over time of tobacco smoke exposure, with the number of cells at each time point (CON, 2M, and 6M) shown at the top of the bars. **c** Bar graphs showing the proportion of γ6δ4 T cells expressing *Il17a* in the air-par, parenchyma and spleen in response to the duration of tobacco smoke exposure. The top of the bars indicates the number of γ6δ4 T cells at the three time points (CON, 2M, and 6M). Respectively, the air-par site is without CON group. CON: air control group, pooled from 3 biological replicates, each comprising cells from 20 mice (total n = 60 mice). 2M: 2-month tobacco smoke exposure group, pooled from 3 biological replicates, each comprising cells from 4 mice (total n = 12 mice). 6M: 6-month tobacco smoke exposure group, pooled from 3 biological replicates, each comprising cells from 4 mice (total n = 12 mice).



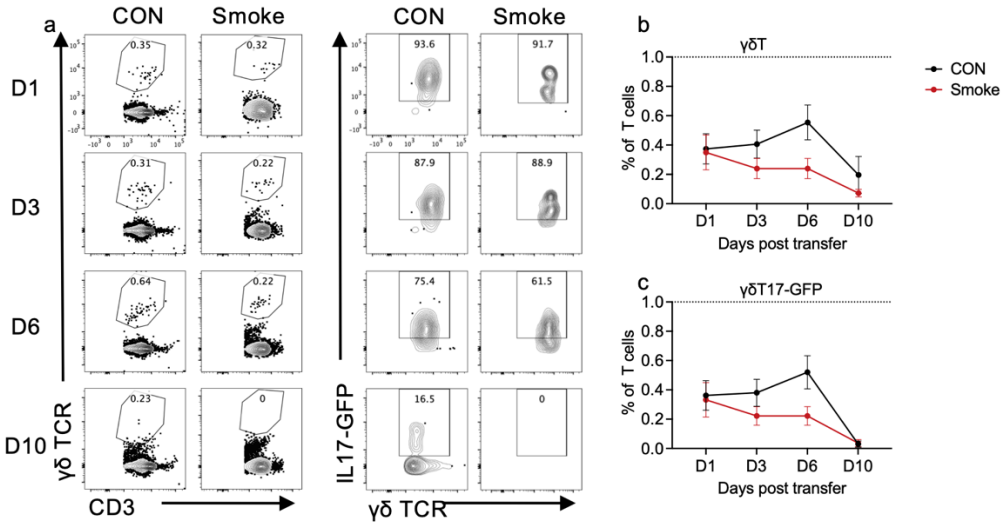
Supplementary Fig. 6. γδ T cells from mice in the air control group and 2mouth or 6 mouth after the tobacco smoke exposure.

Cell clustering from single-cell whole transcriptome analysis (WTA) data was performed on 5295 γδ T cells from total T cells, with 1120 cells from airways, 1920 cells from air-par, 2039 cells from parenchyma, and 216 cells from spleen from air control (CON, pooled from $n = 3$, mixed from 60 mice), tobacco smoke-exposed for 2 months (2M, pooled from $n = 3$, mixed from 12 mice), and tobacco smoke-exposed for 6 months (6M, pooled from $n = 3$, mixed from 12 mice) C57BL/6J male mice. **a** UMAP showing the scRNA-seq clusters of 5295 γδ T cells. **b** Differential representation of γδ T cell clusters. **c** Scaled gene expression. CON: air control group; 2M: 2-month tobacco smoke exposure group; 6M: 6-month tobacco smoke exposure group.



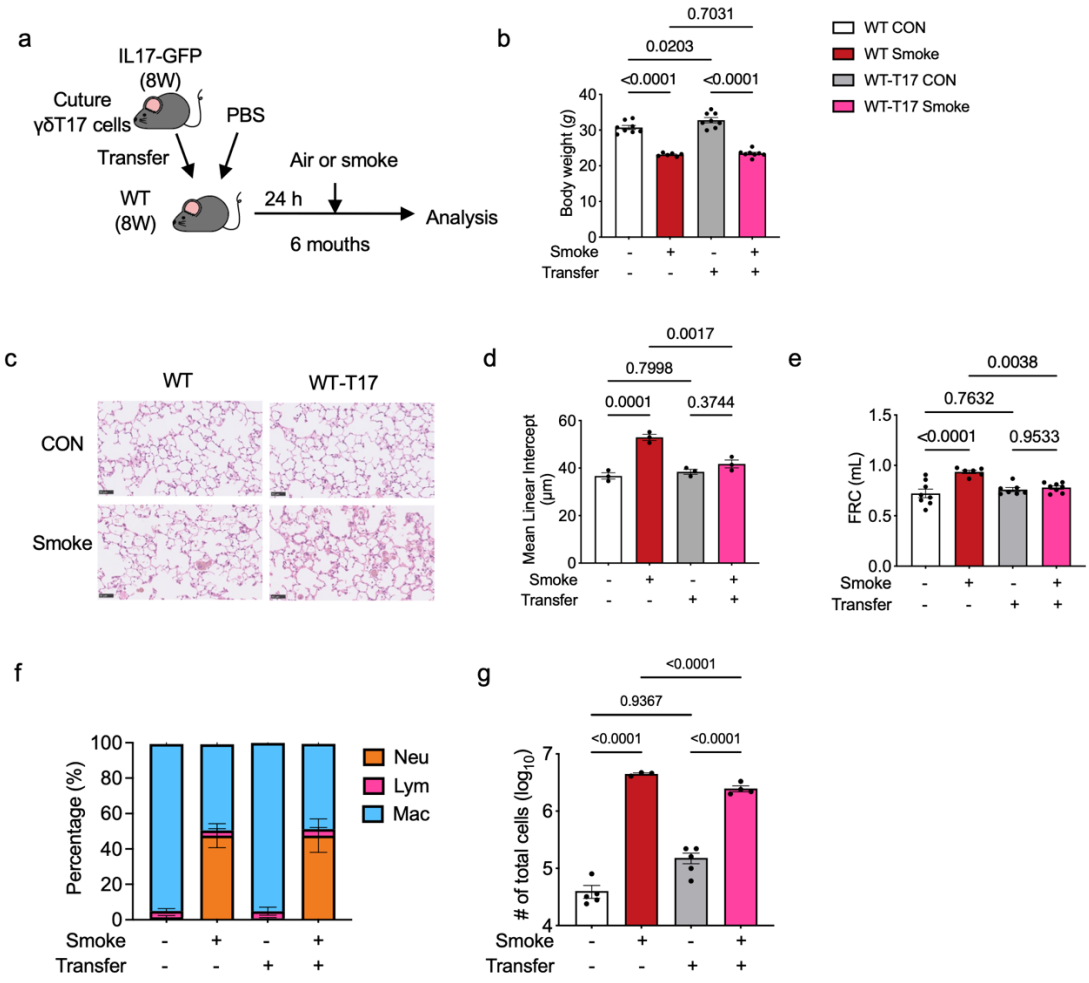
Supplementary Fig. 7. In vitro expansion of γδT17 cells.

a Schematic of culture protocol. **b-e** Representative flow cytometry and number and frequency of in vitro-expanded γδT17 cells at different stages of culture (n = 3).



Supplementary Fig. 8. *Tcrd*$^{-/-}$ mice in air control group (CON) and smoke exposure group (Smoke) were tansfered with γδT17 cells.

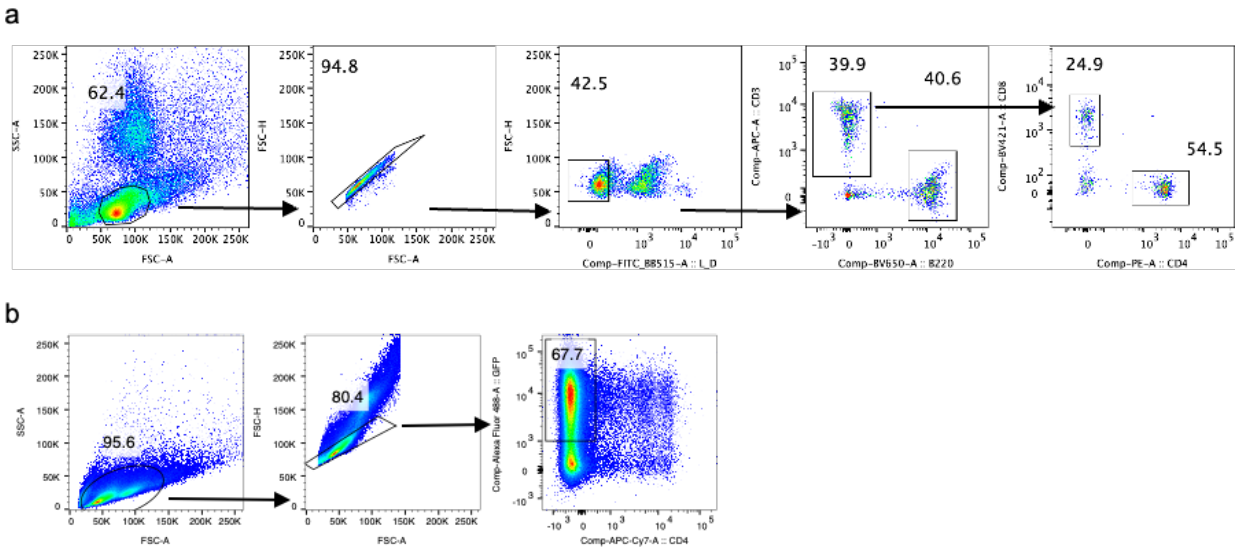
a Representative flow cytometry of γδ T cells and γδ T17 cells from blood of *Tcrd*$^{-/-}$ mice. **b-c** The percentage of γδ T cells (**b**) and γδ T17 cells (**c**) from blood were monitored at the indicated time points post transfer. *n* = 5 mice/group/time point.



Supplementary Fig. 9. γδ T17 cells ameliorate emphysema-like phenotype and airway inflammations.

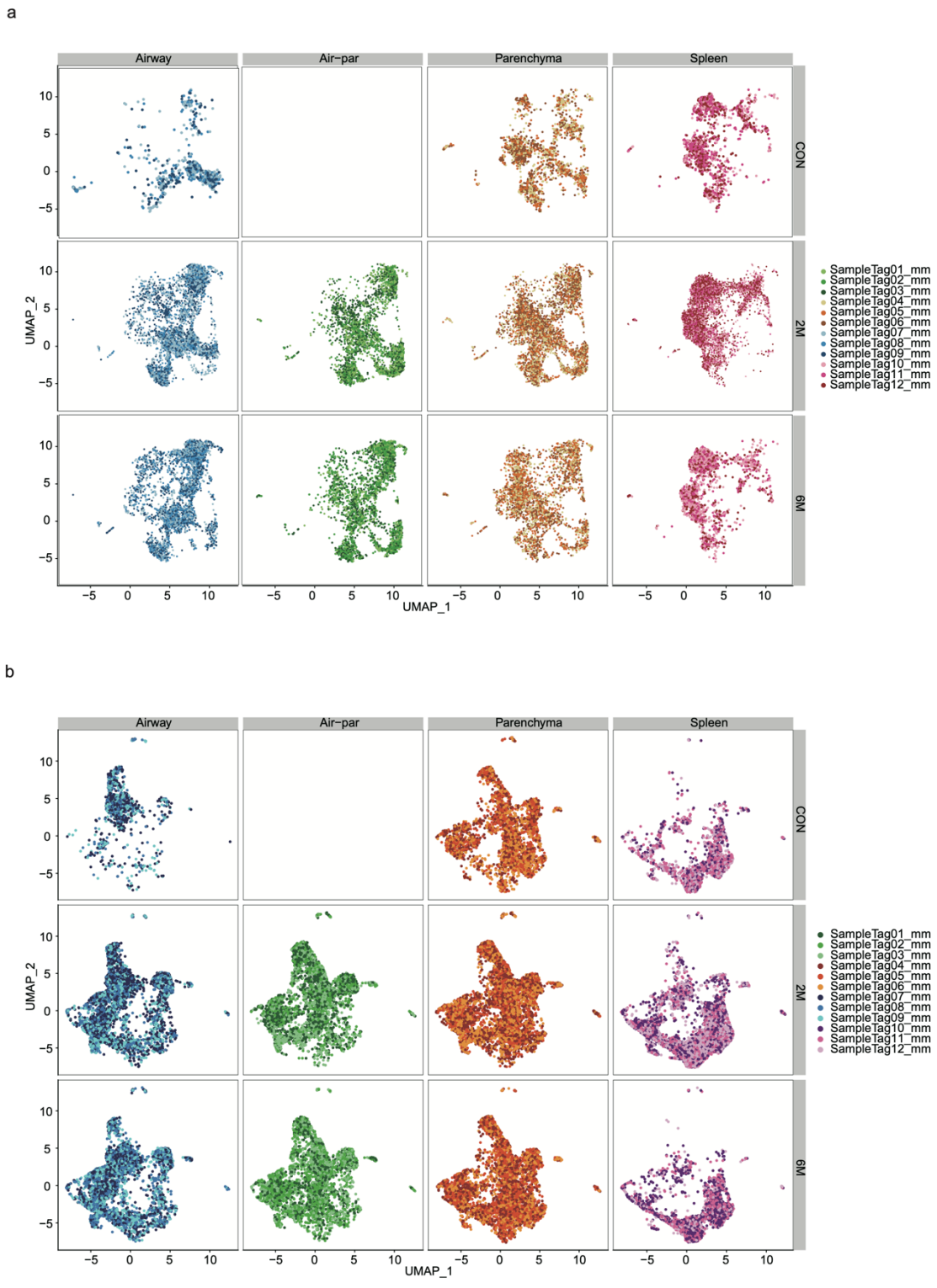
a, b C57BL/6J wild-type (WT) mice were randomly divided into two groups: the air control group (CON) and the tobacco smoke exposure group (Smoke). Mice were placed in a fume box and exposed to tobacco smoke for 6 months, respectively. Twenty-four hours prior to tobacco smoke exposure, mice were randomly divided into two groups: the transferred γδT17 cell (T17) group and the PBS control group, with body weights recorded at 6 months (6M), after exposure (WT CON group, *n* = 8 mice; WT smoke group, *n* = 6 mice; WT-T17 CON groups, *n* = 8 mice; WT-T17 smoke groups, *n* = 8 mice). **c** Representative images of hematoxylin and eosin-stained paraffin sections (40 ×, scale bar = 50 μm) of the WT, and WT-T17 groups show that after 6 months of tobacco smoke exposure the Smoke group exhibited marked airway inflammation and fused

alveolar damage compared to the CON group. The experiment was repeated independently at least three times with similar results. **d** Mean linear intercept (MLI) values are presented (n = 3 mice/group). **e** Functional residual capacity (FRC) measurements are shown (WT CON group, n = 8 mice; WT smoke group, n = 6 mice; WT-T17 CON groups, n = 7 mice; WT-T17 smoke groups, n = 8 mice). **f, j** The proportion (**f**) and absolute counts (**g**) of inflammatory cells in bronchoalveolar lavage fluid (BALF) are shown (WT CON group, n = 5 mice; WT smoke group, n = 3 mice; WT-T17 CON groups, n = 5 mice; WT-T17 smoke groups, n = 4 mice). Data are presented as mean ± SEM. One-way ANOVA was used for comparisons among multiple groups when data were normally distributed, while the non-parametric Kruskal–Walli's test was applied for non-normally distributed data.



Supplementary Fig. 10. Gating strategy.

a Gating strategy for analyzing T cells in mouse model. **b** Gating strategy for sorting γδT17 cells after in vitro expansion.



Supplementary Fig. 11. T Cells from different sample tags.

a, b UMAP Plots showing T cells labeled by different sample tags in (**a**) single-cell targeted transcriptome analysis (TTA) data and (**b**) single-cell whole transcriptome analysis (WTA) data.

Supplementary Table 1. The key reagent used for flow cytometry, cell culture and BD Rhapsody sequencing.

REAGENT or RESOURCE	SOURCE	Cat#
Antibodies		
APC anti-mouse CD3ε antibody, clone 145-2C11, 1:100 dilution	Biolegend	100312
PE-Cy7 anti-mouse CD3e, clone 145-2C11, 1:100 dilution	BD Biosciences	552774
PE anti-mouse CD4 antibody, clone GK1.5, 1:100 dilution	Biolegend	100408
eFluor 450 anti-mouse CD8a monoclonal antibody, clone 53-6.7, 1:100 dilution	eBioscience	47-0081-82
BV650 Anti-mouse CD45R/B220, clone RA3-6B2, 1:100 dilution	BD Biosciences	563893
APC-Cy7 anti-mouse CD4, clone GK1.5, 1:100 dilution	BD Biosciences	552051
APC anti-mouse TCR γ/δ antibody, clone GL3, 1:100 dilution	bioLegend	118116
APC-eFluor 780 anti-mouse CD90.2 (Thy-1.2) antibody, clone 53-2.1, 0.5mg per mouse	eBioscience	47-0902-82
Brilliant Violet 510 anti-mouse CD45 antibody, clone 30-F11, 0.25mg per mouse	Biolegend	103138
InVivoMAb anti-mouse TCR γ/δ, clone UC7, 10μg per milliliter	Bioxcell	BE0070
InVivoPlus anti-mouse IFNγ, clone XMG1.2, 10μg per milliliter	Bioxcell	BP0055
Chemicals and Recombinant Proteins		
Collagenase D	Roche	11088882001
DNase I	Roche	10104159001
FBS	Excell bio	FSD500
MEM Non-Essential Amino Acids Solution (100X)	Gibco	11140035
L-glutamine	Gibco	A2916801
HEPES	Gibco	15630130
Pen Strep (5,000 U/mL)	Gibco	15070063
2-Mercaptoethanol	Gibco	21985023
Sodium Pyruvate(100 mM)	Gibco	11360070
HBSS with Ca^{2+} Mg^{2+}	Gibco	14025076
Recombinant mouse IL-23 (carrier-free)	Biolegend	589004
Recombinant mouse IL-7 (carrier-free)	Biolegend	577804
Recombinant murine IL-1β	Perprotech	211-11B
Critical Commercial Assays		
BD Rhapsody Cartridge Kit	BD Biosciences	633733
BD Rhapsody Cartridge Reagent Kit	BD Biosciences	633731
BD Rhapsody cDNA Kit	BD Biosciences	633773
BD Rhapsody WTA Amplification Kit	BD Biosciences	633801
BD Rhapsody Immune Response Panel Mm	BD Biosciences	633753
BD Mouse Single-Cell Sample Multiplexing Kit	BD Biosciences	626545
Qubit dsDNA HS Assay Kits	Thermo Fisher Scientific	Q32851
Fixable Viability Dye eFluor 520	eBioscience	65-0867-14

Supplementary Table 2. Sample tag correspondence.

	CON	2M	6M
Sample tag1		Air-par1	Air-par1
Sample tag2		Air-par2	Air-par2
Sample tag3		Air-par3	Air-par3
Sample tag4	Parenchyma1	Parenchyma1	Parenchyma1
Sample tag5	Parenchyma2	Parenchyma2	Parenchyma2
Sample tag6	Parenchyma3	Parenchyma3	Parenchyma3
Sample tag7	Airway1	Airway1	Airway1
Sample tag8	Airway2	Airway2	Airway2
Sample tag9	Airway3	Airway3	Airway3
Sample tag10	Spleen1	Spleen1	Spleen1
Sample tag11	Spleen2	Spleen2	Spleen2
Sample tag12	Spleen3	Spleen3	Spleen3