SUPPLEMENTAL MATERIALS

Supplemental Materials

Tables

Figures

Table S1. Tissues collected at necropsy

Animal identification
Adrenal glands
Aorta
Bone with articular surface and marrow (femur)
Brain
Esophagus
Eyes with optic nerves
Gallbladder
Gross lesions
Heart
Intestine, large (cecum, colon, rectum)
Intestine, small (duodenum, jejunum, ileum)
Kidneys
Larynx (3 sections)
Liver (median lobe and left lateral lobe)
Lungs with bronchia
Lymph node (mandibular, mesenteric, tracheobronchial)
Mammary gland

Nasal turbinates (4 levels)
Ovaries (without oviduct)
Pancreas
Pharynx
Pituitary gland
Salivary gland (mandibular)
Sciatic nerve
Skeletal muscle (biceps femoris)
Spinal cord (thoracolumbar)
Spleen
Stomach
Thymus
Thyroid gland (with parathyroids, if present in routine section)
Trachea
Tongue
Urinary bladder
Uterus (with cervix)

Table S2. Body weight and food consumption

Parameter	Group				
	Sham (Air) Control (0 µg TPM/L)	Carrier (1,550 µg TPM/L)	Test-1 (1,400 µg TPM/L)	Test-2 (1,400 µg TPM/L)	3R4F (550 µg TPM/L)
Body Weight (mean [g] ± SD)					
Day -7*	20.282±2.584	21.008 ±1.404	20.905±1.701	21.119±1.197	20.773±1.949
Day 1*	20.593 ±1.044	20.468±1.049	20.890±1.222	20.656±1.227	20.921±1.125
Day 5**	20.080±1.025	19.963±1.109	20.020±1.156	20.048±1.053	19.839±1.216
Day 8*	21.120±1.077	20.866±1.211	20.971±1.202	20.631±1.103	21.119±1.260
Day 12***	19.976±1.045	20.110±1.177	20.202±1.308	19.600±1.877	19.712±1.257
Day 15***	21.317±1.120	21.455±1.100	21.695±1.161[c]	21.407±1.498	21.051±1.234
Day 17[&]	20.517±0.954	20.883± 0.983	19.933±1.164	20.533±1.500	20.067± 1.076
Day 18[@]	19.840±1.265	20.133±1.072	20.160±1.331	20.820±1.195[c]	19.533±0.756
Day 19[#]	20.580±0.846	20.726±1.474	21.095±1.298	19.925±1.740[d]	20.505±1.213
Body Weight Gain (mean [g] ± SD/day)*					
Day 1*	0.039±0.291	-0.068±0.140	-0.002±0.155 [b]	-0.058±0.096	0.019±0.249
Day 5**	-0.128±0.231	-0.126±0.194[c]	-0.218±0.141[a,b]	-0.152±0.172[c,d]	-0.271±0.200[a]
Day 8 **	0.346±0.200	0.301±0.222	0.317±0.224	0.194±0.154[a,b,c,d]	0.414±0.296
Day 12***	-0.286±0.200	-0.188±0.141[c]	-0.192±0.246[c]	-0.258±0.413[c]	-0.352±0.217
Day 15***	0.447±0.229	0.448±0.237	0.498±0.265	0.602±0.599	0.446±0.279
Day 17[&]	-0.817±0.413	-0.675±0.238	-0.950±0.449[c]	-0.650±0.202	-0.392±0.139
Day 18[@]	-0.393±0.356	-0.300±0.239	-0.420±0.168	-0.353±0.228	-0.387±0.225
Day 19[#]	-0.178±0.183	-0.204±0.233	-0.191±0.100	-0.250±0.568	-0.219±0.169
Feed Consumption (mean [g] ± SD/day)					
Day 8[*]	4.0±0.4	3.7±0.4[a]	4.0±0.6[c]	3.9±0.5	3.7±0.7[a]
Day 15[%]	4.1±0.4	3.9±0.8[a,c]	4.1±0.9[c]	3.8±0.6[a,c]	3.6±1.2[a]
Day 17[&]	3.4±0.3	3.3±0.5[c]	3.2±0.3[c]	2.8±0.3[a]	2.6±0.2[a]
Day 18[@]	3.4±0.5	3.5±0.4[c]	3.4±0.5[c]	3.3±0.4[c]	2.7±0.3[a]
Day 19[#]	3.7±0.4	3.6±0.3[c]	3.8±0.4[c]	3.5±0.9[c]	2.9±0.3[a]

*N=41 all Groups except N=39 in Sham (Air) group for food consumption Day 8

**N=41 all Groups except N=39 for 3R4F Cigarette Group

***N=41 all Groups except N=40 for Carrier Group

[&]N=6 all Groups

[$]N=41 all Groups except N=39 in Sham (Air) group

[%]N=41 all Groups except N=39 for Carrier Group and N=40 for Test-1 Group

[@]N=15 all Groups

[#]N=20 all Groups except N=19 for Carrier Group

[a] $p \leq 0.05$ compared to Sham (Air) Control

[b] $p \leq 0.05$ compared to Carrier

[c] $p \leq 0.05$ compared to 3R4F cigarette

[d] $p \leq 0.05$ compared to Test-1

Table S3. BALF (first wash) parameters

MAP-MMP-PRM
satistics

Multi-Analyte Profiling (MAP)

Kit	Provider	Catalog number	Markers
Mouse Cytokine/ Chemokine Panel I	EMD Millipore	MCYTOMAG-70K-PMX	G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES, TNF-α
Mouse CVD1	EMD Millipore	MCVD1MAG-77K	proMMP-9, PAI-1 (total), sE-selectin, sICAM-1,thrombomodulin, pecam-1, sP-selectin

Abbreviations

- G-CSF, Granulocyte colony-stimulating factor
- IL, Interleukin
    - IL-7, IL-4, IL-1a, IL-9, IL-10, IL-12, IL-6, IL-7, IL-5
- IP-10, interferon γ-induced protein 10 kDa
- KC, Keratinocyte chemoattractant
- MCP-1, Monocyte Chemoattractant Protein-1
- MIP, Macrophage inflammatory protein
    - MIP-1a, MIP-1b, MIP-2
- TNF, Tumor necrosis factor
- proMMP-9, Precursor of matrix metalloproteinase 9
- GM-CSF, Granulocyte-macrophage colony-stimulating factor

- aldehyde dehydrogenase 3 family member A1 (AL3A1)
- apolipoprotein A1 (ApoA1)
- cathepsin D (CATD)
- cathepsin S (CATS)
- chemokine (C-X-C motif) ligand 15 (CXCL15)

- fetuin-A (FETUA)
- fibrinogen beta chain (FIBB)
- SERPING1 (IC1)
- intercellular adhesion molecule 1 (ICAM-1)
- putative permease (PERM)
- surfactant protein D (SFTPD)
- superoxide dismutase (SODE)
- uteroglobin (UTER), and
- von Willebrand factor (vWF)

As appropriate, data are plotted in Tables S7 and S9 - by treatment group, using mainly boxplots for the univariate illustration of the studied endpoints. The logarithmic scale was used for illustration purposes to facilitate group comparisons. Multivariate charts (principal component biplots) were also used to visualize all analyzed tissue samples vs. all targeted endpoints.

Table S4. BALF parameters: cytology

Parameter	Group				
	Sham (Air) Control (0 µg TPM/L)	Carrier (1550 µg TPM/L)	Test-1 (1400 µg TPM/L)	Test-2 (1400 µg TPM/L)	3R4F (550 µg TPM/L)
Bronchoalveolar Lavage – Flow Cytometry – 3 Week Sacrifice* (mean [cells x10³ per mL] ± SD)					
Live Cells	219±802	194±86[c]	269±292[c]	220±56[c]	556±94 (N=14)
Dead Cell	27±14	25±7[c]	34±15[c]	45±26[a,b,c]	351±71[a] (N=14)
Total CD45+ Leukocytes	160±65	138±82[c]	135±41[c]	161±52[c]	417±87 [a] (N=14)
Total Macrophages	195±74	172±82[c]	162±40[c]	185±54[c]	38±12 [a] (N=14)
Total Alveolar Macrophages	194±74	171±82[c]	161±39[c]	180±57 [c]	18±4 [a] (N=14)
Total Interstitial Macrophages/Monocytes	1±0.3 (N=8)	3±2[c] (N=7)	2±3[c] (N=9)	6±12[a,c] (N=12)	19±10[a] (N=14)
Dendritic Cells	1 (N=1)	ND	1±1[c] (N=3)	3±2[c] (N=4)	20±8[a] (N=14)
Total activated (CD54+) Macrophages/Monocytes	14±8	9±3	15±16	10±3	10±3 (N=14)
Activated (CD54) Alveolar Macrophages	14±8	9±3	11±7	10±3	8±2 (N=14)
Activated (CD54) Interstitial Macrophages/Monocytes	0.06±0.03 (N=8)	0.06±0.02[c] (N=7)	0.08±0.08[c](N=9)	0.2±0.3[c] (N=12)	3±1 (N=14)
Activated (CD54+) Dendritic Cells	0.07 (N=1)	0.08 (N=1)	0.03±0.03[c] (N=3)	0.1±0.1[c] (N=4)	0.7±0.6 (N=14)
MHC II High Alveolar Macrophages	8±4	8±4[c]	9±6[c]	7±5[c]	14±3 (N=14)
MHC II High Interstitial Macrophages/Monocytes	0.2±0.2 (N=8)	0.1±0.07[c] (N=7)	0.2±0.2[c] (N=9)	0.4±0.3[c] (N=12)	9±3 (N=14)
MHC II High Dendritic Cells	0.14 (N=1)	0.3 (N=1)	0.09±0.06[c] (N=3)	0.2±0.2[c] (N=4)	3±1 (N=14)
Total Granulocytes	2 (N=1)	ND	ND	ND	238±42 (N=14)
Neutrophils	2 (N=1)	ND	ND	ND	230±40 (N=14)
Eosinophils/Basophils/Mast Cells	ND	ND	ND	ND	9±4 (N=11)
Total activated (CD54+) Granulocytes	ND	ND	ND	ND	2±0.9 (N=14)
Activated (CD54+) Neutrophils	0.07 (N=1)	ND	ND	ND	2±0.8 (N=14)
Activated (CD54+) Eosinophils/Basophils/Mast Cells	ND	ND	ND	ND	0.1±0.2 (N=11)
B-Cells	2 ±1	3 ±1[c]	2±1[c]	3±2[c]	22±12[a] (N=14)
Activated (CD69+) B-Cells	0.6±0.4(N=12)	0.6±0.4[c]	0.7±0.5[c](N=13)	0.7±0.7[c] (N=13)	7±4 (N=14)
MHC II High B-Cells	0.2±0.2 (N=12)	0.4±0.6[c]	0.4±0.3[c] (N=13)	0.4±0.5[c] (N=13)	13±9 (N=14)
Total CD3+ T-Cells	7±5	6±6[c]	5±4[c]	5±3[c]	150±54[a] (N=14)
CD4+ T-Cells	3±2 (N=5)	1 (N=1)	4±6[c] (N=5)	2±3[c] (N=2)	44±24[a] (N=14)
CD8+ T-Cells	4±1 (N=6)	8±9[c] (N=3)	4±2[c] (N=4)	5±4[c] (N=4)	93±37[a] (N=6)
Activated (CD69+) CD4+ T-Cells	0.9±0.5 (N=5)	0.2 (N=1)	0.7±0.6[c] (N=5)	1±2[c] (N=2)	19±9 (N=14)
Activated (CD69+) CD8+ T-Cells	4±1 (N=6)	8±9[c] (N=3)	3±2[c] (N=4)	4±4[c] (N=4)	85±36 (N=6)

[a] $p \leq 0.05$ compared to Sham (Air) Control;

[b] $p \leq 0.05$ compared to compared to Carrier;

[c] $p \leq 0.05$ compared to 3R4F cigarette;

[d] $p \leq 0.05$ compared to Test-1;

*N=15 per group (the N used for summary statistics differs per parameter based on if enough events of the parent population of cells could be counted during analysis; Actual N (if not 15) are included

ND = Not detected
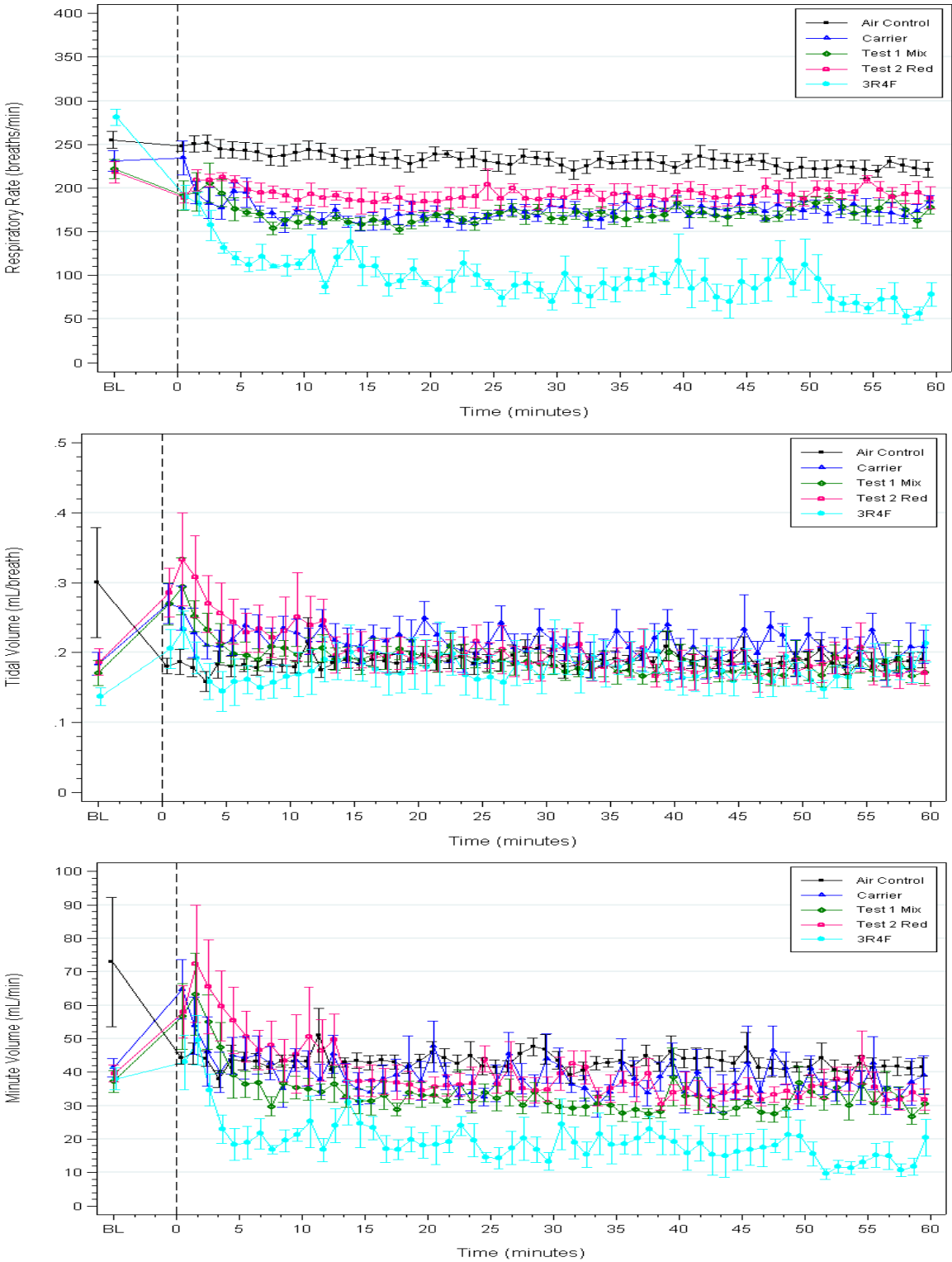
Figure S1. Respiratory physiology

[SEPARATE PDF]

Figure S2. BALF (first wash): MAP

Boxplots for selected BALF cytokines for the study groups (from left to right: Sham, Carrier, Test-1, Test-2, and 3R4F). Black thick lines inside the boxes represent the sample medians. Box's lower and upper limits depict the first and the third sample quartiles, respectively. Box whiskers are defined by extending the box ends by a multiple of the data variability. Points not included within the whiskers are depicted by black dots and may be outliers. Data are plotted on the logarithmic scale for easier visualization and group comparisons. The y-axis on the left reports values in the logarithmic scale, while on the right reports values in the original scale. Red and green dashed-dotted lines illustrate the limits of quantification and detection, respectively. The descriptive statistics are given in Figure S3.

[SEPARATE PDF]

Figure S3. BALF (first wash): PRM

Parallel reaction monitoring (PRM) of BALF cytokines (The used protein sequence is given next to the protein name in the header): Boxplots for selected parameters for the study groups (from left to right: Sham, Carrier, Test-1, Test-2, and 3R4F). Black thick lines inside the boxes represent the sample medians. Box's lower and upper limits depict the first and the third sample quartiles, respectively. Box whiskers are defined by extending the box ends by a multiple of the data variability. Points not included within the whiskers are depicted by black dots and may be outliers. Data are plotted on the logarithmic scale for easier visualization and group comparisons. The y-axis on the left reports values in the logarithmic scale, while on the right reports values in the original scale. The descriptive statistics are given Figure S3.
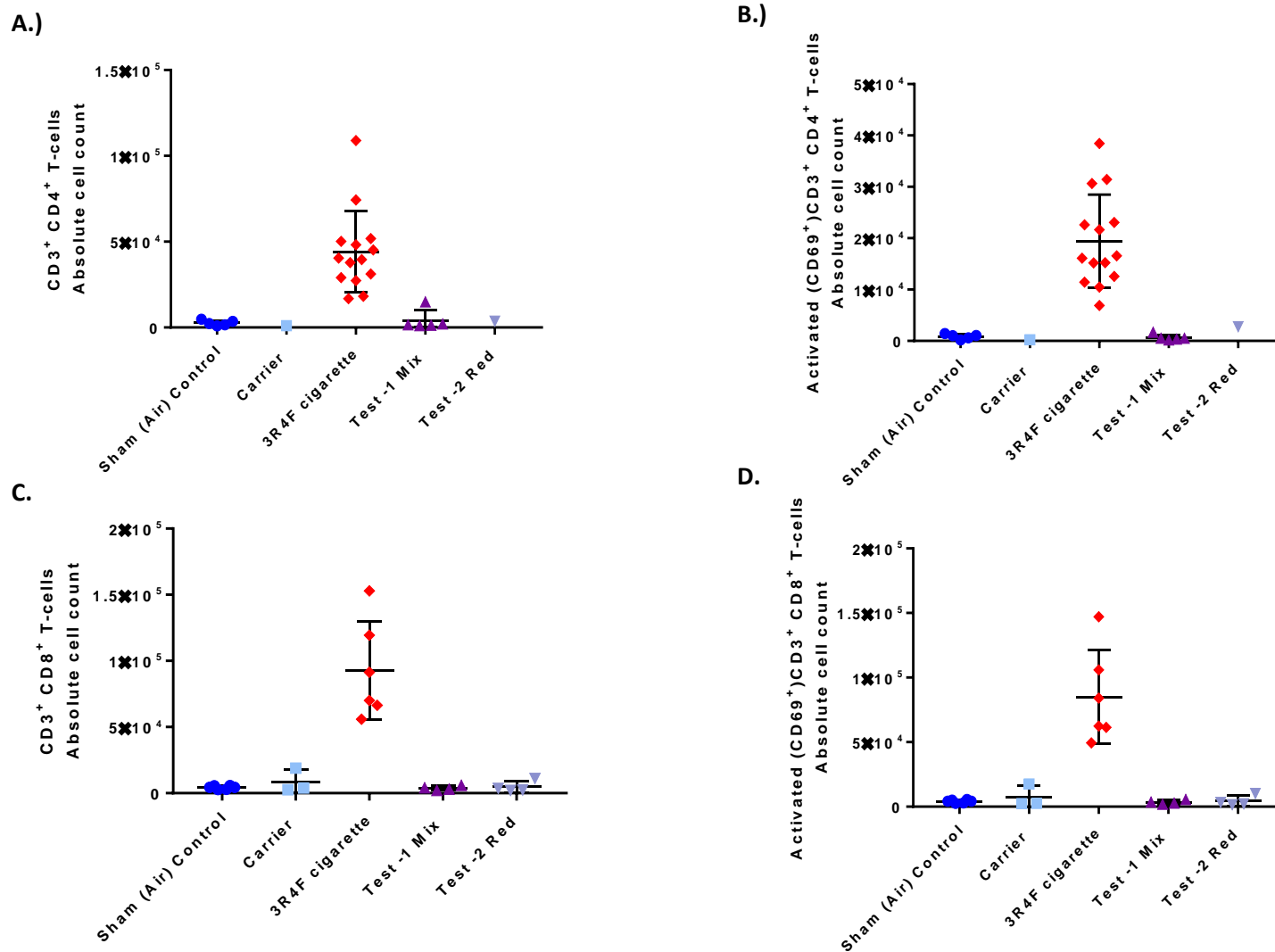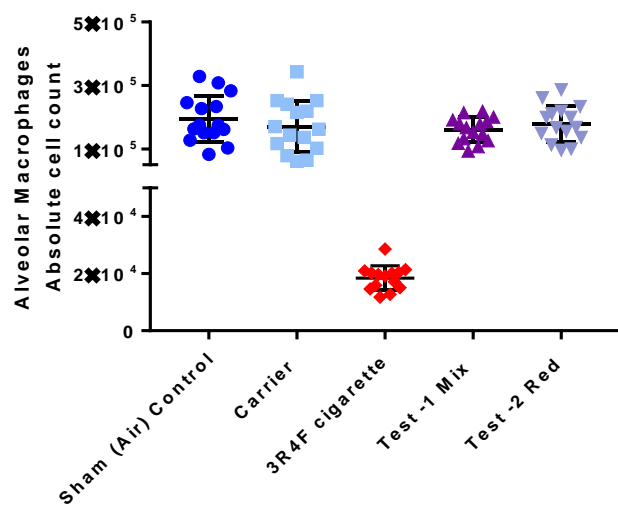
Figure S4. BALF cytology: T-cells

A) Number of CD3^{+} CD4^{+} T-cells recovered after bronchial lavage

B) Number of activated (CD69^{+}) CD3^{+} CD4^{+} T-cells recovered after bronchial lavage

C) Number of CD3^{+} CD8^{+} T-cells recovered after bronchial lavage

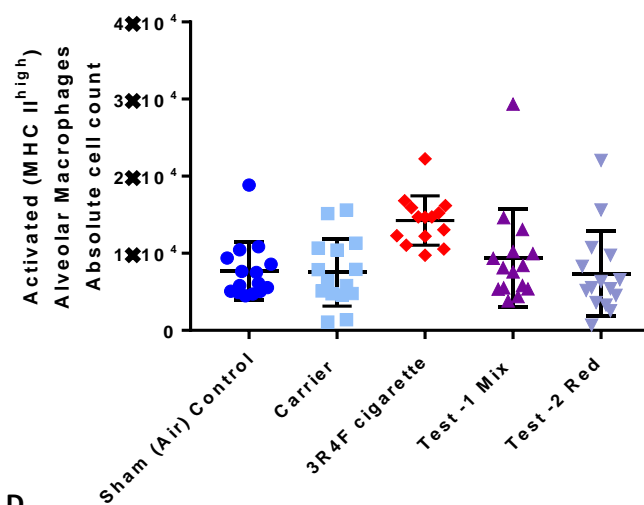**D)** Number of activated (CD69^{+}) CD3^{+} CD8^{+} T-cells recovered after bronchial lavage
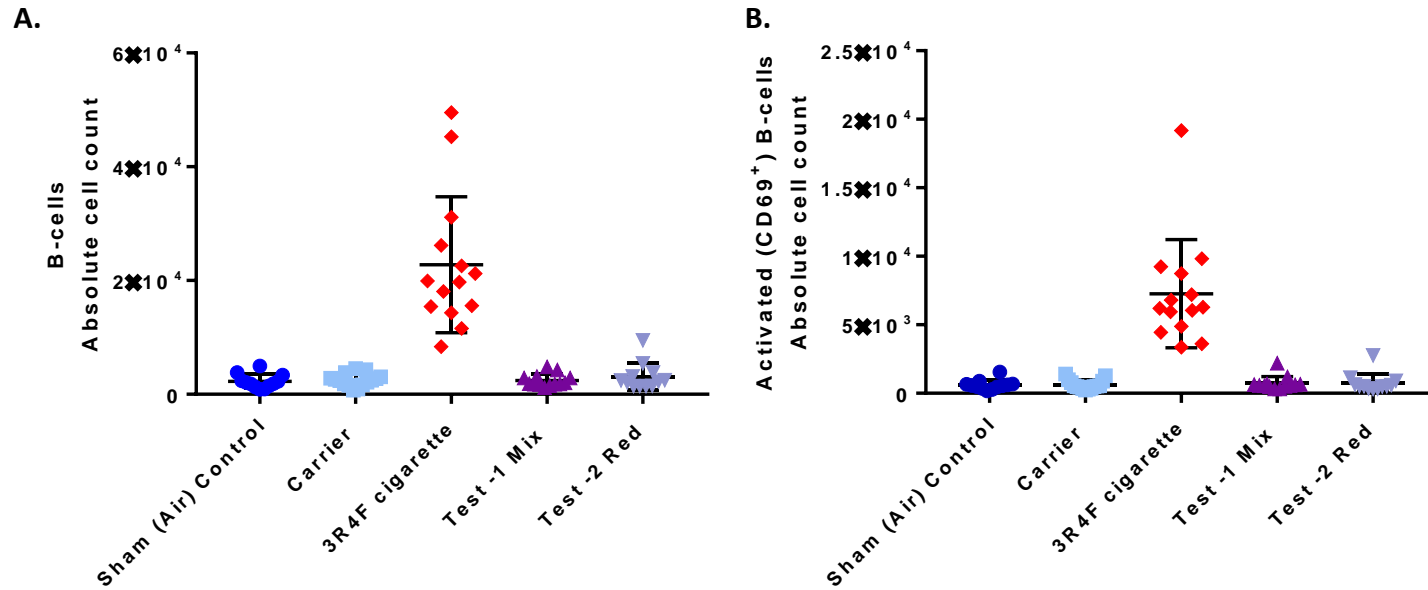
Figure S5. BALF cytology: B-cells

A) Number of B-cells recovered after bronchial lavage.
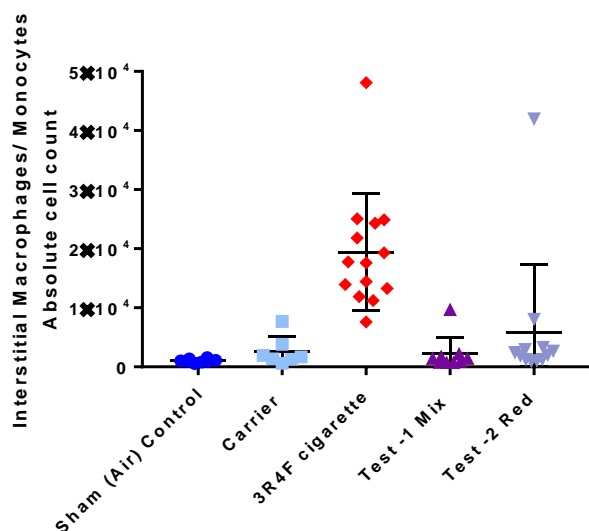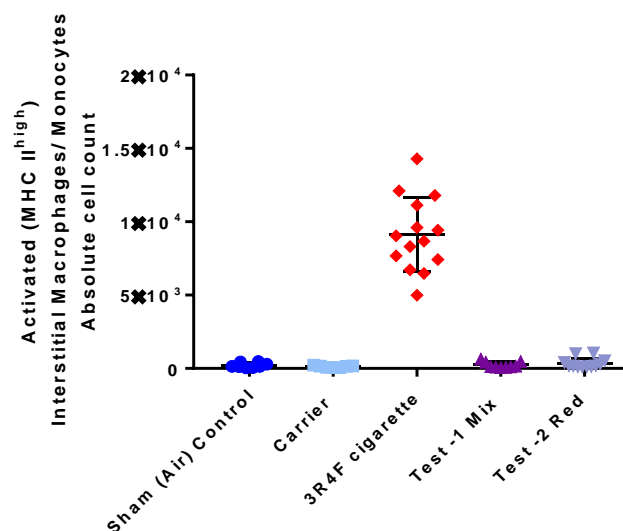**B)** Number of activated (CD69^{+}) B-cells recovered after bronchial lavage.
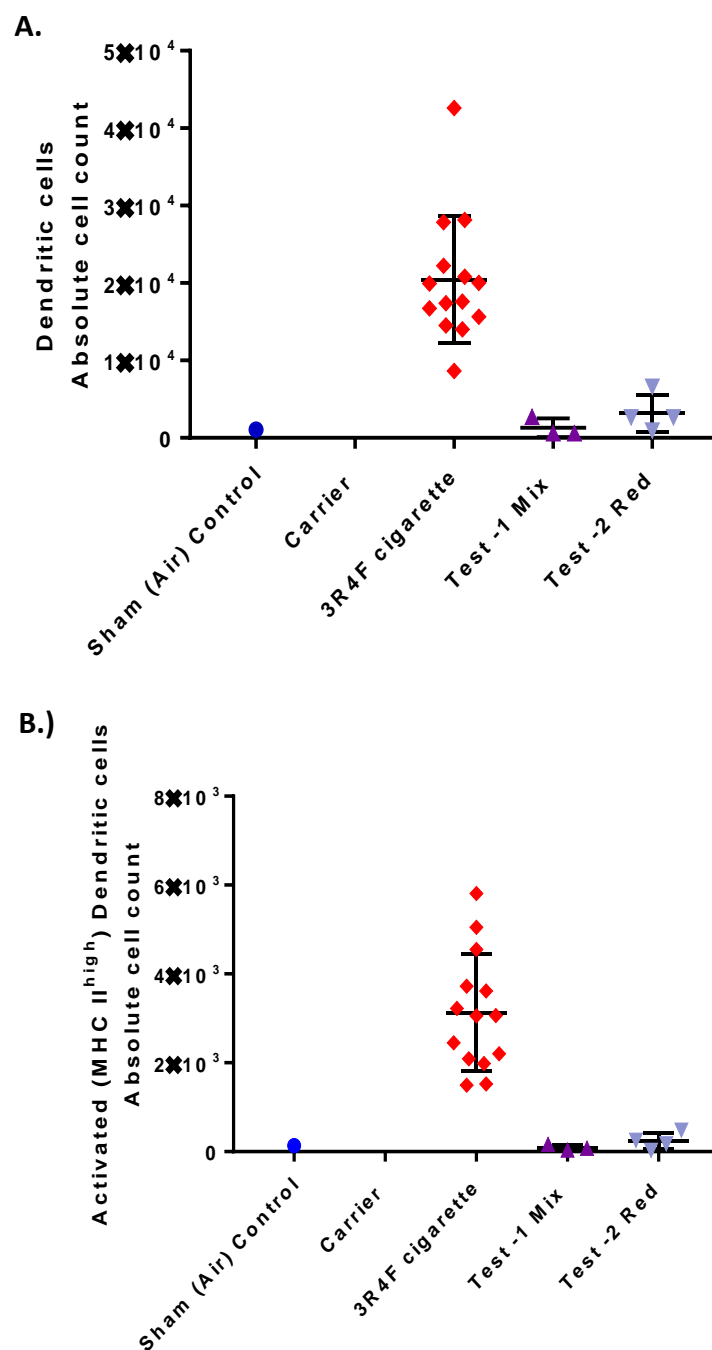
A.

B.

C.

D.

Figure S6. BALF cytology: macrophages and monocytes

A) Number of Alveolar Macrophages recovered after bronchial lavage

B.) Number of activated (MHC II[high]) Alveolar Macrophages recovered after bronchial lavage

**C.)** Number of Interstitial Macrophages/Monocytes recovered after bronchial lavage

D.) Number of activated (MHC II[high]) Interstitial Macrophages/Monocytes recovered after bronchial lavage

A.

B.)

Figure S7. BALF cytology: dendritic cells

A) Number of Dendritic cells recovered after bronchial lavage
B) Number of activated (MHC II^{high}) Dendritic cells recovered after bronchial lavage

Alveolar Macrophages
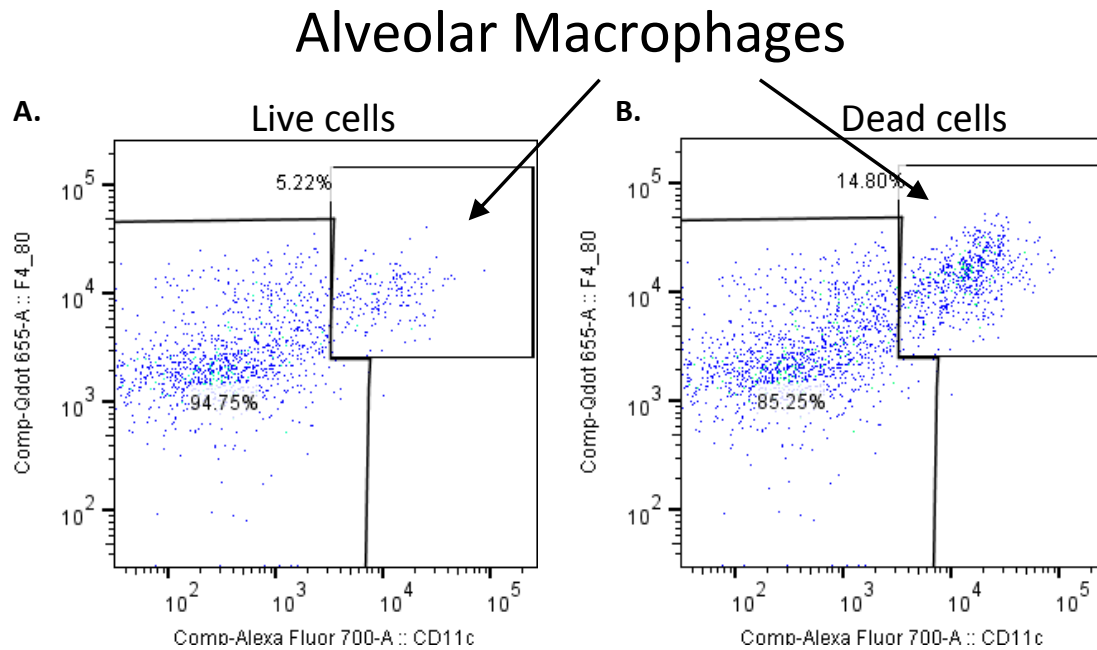
Figure S8. BALF cytology: density plots

Example live and dead cell populations gated for BALF alveolar macrophages from the 3R4F group
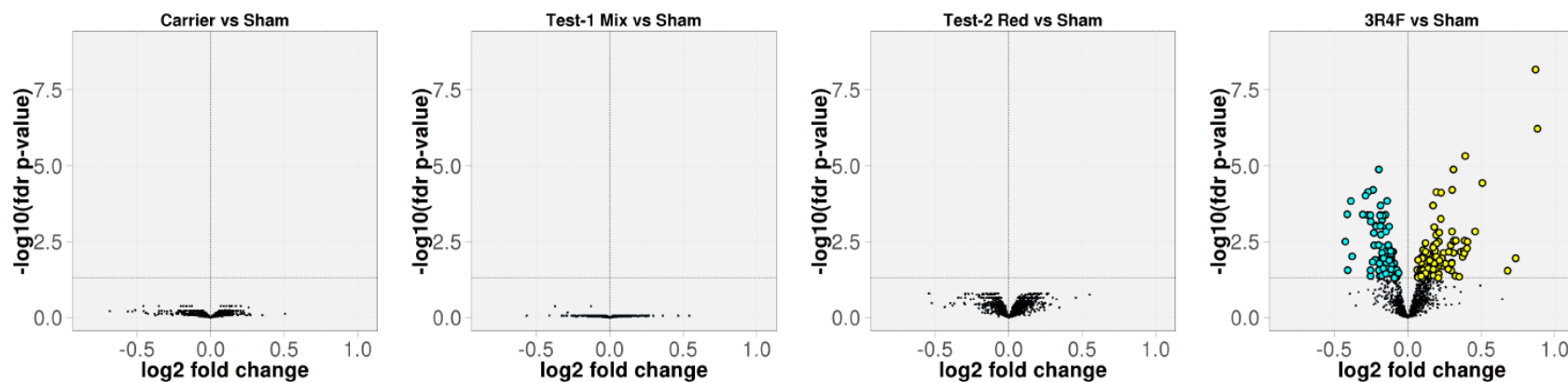
Figure S9. Volcano plots representing the changes in protein abundances in the lung

Volcano plots representing the protein response profiles in the lung. For each protein, the protein expression change, calculated as the log2 fold-change, is plotted on the x-axis and the statistical significance, proportional to the negative log10-adjusted p-value, is plotted on the y-axis. Yellow and blue dots highlight proteins that are statistically significantly up- and down-regulated, respectively

[SEPARATE PDF]

Figure S10. Comparison of differentially expressed genes between the contrasts.

A) Differentially expressed genes in common based on FDR p-value <0.05. Correlation (Cor) coefficients are color coded (see key), the numbers of overlapping differentially abundant genes are indicated in each cell (with consistency of the change in direction indicated by the pie chart; see key), green stars indicate significance of a Fisher association test (p < 0.05).

B) Heatmap of the gene fold-changes (log2, normalized to the maximum observed absolute value) which are significant (FDR<0.05) in at least one comparison.