Supplemental Figure 1. R.C.A. de Groot et al.

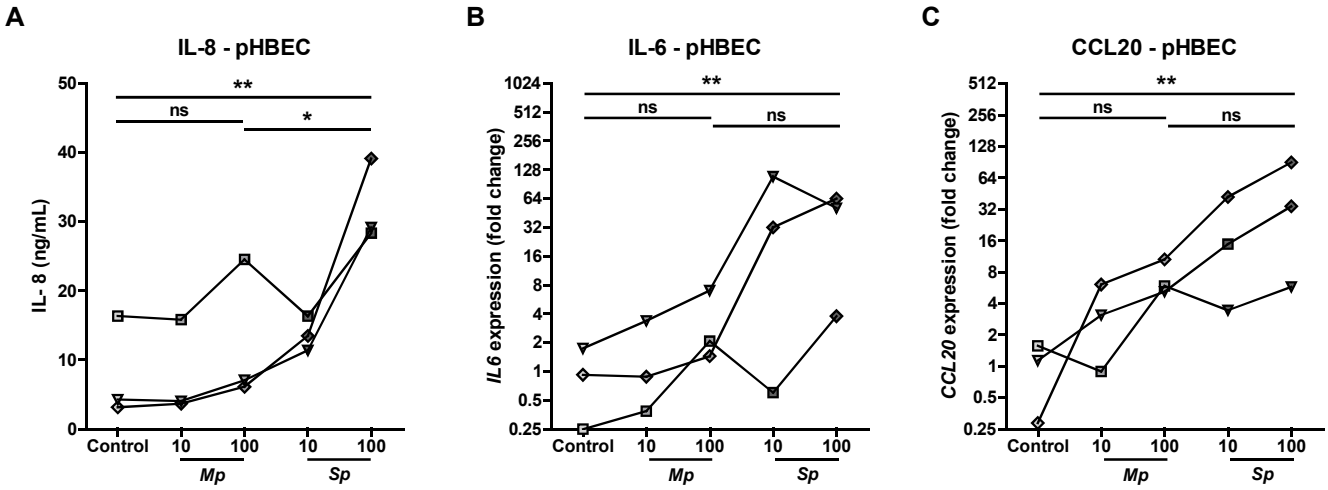


Figure 2. R.C.A. de Groot et al.

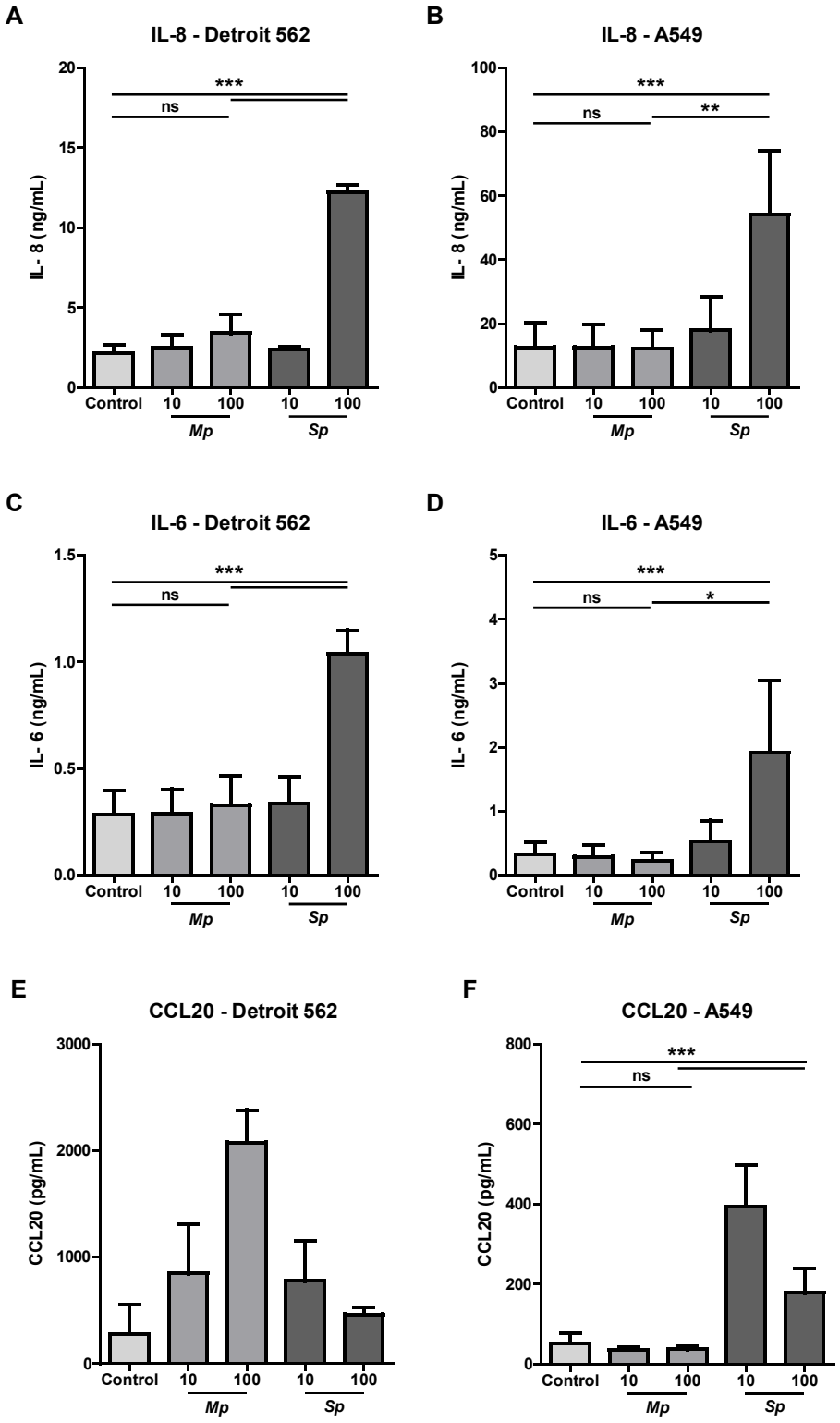
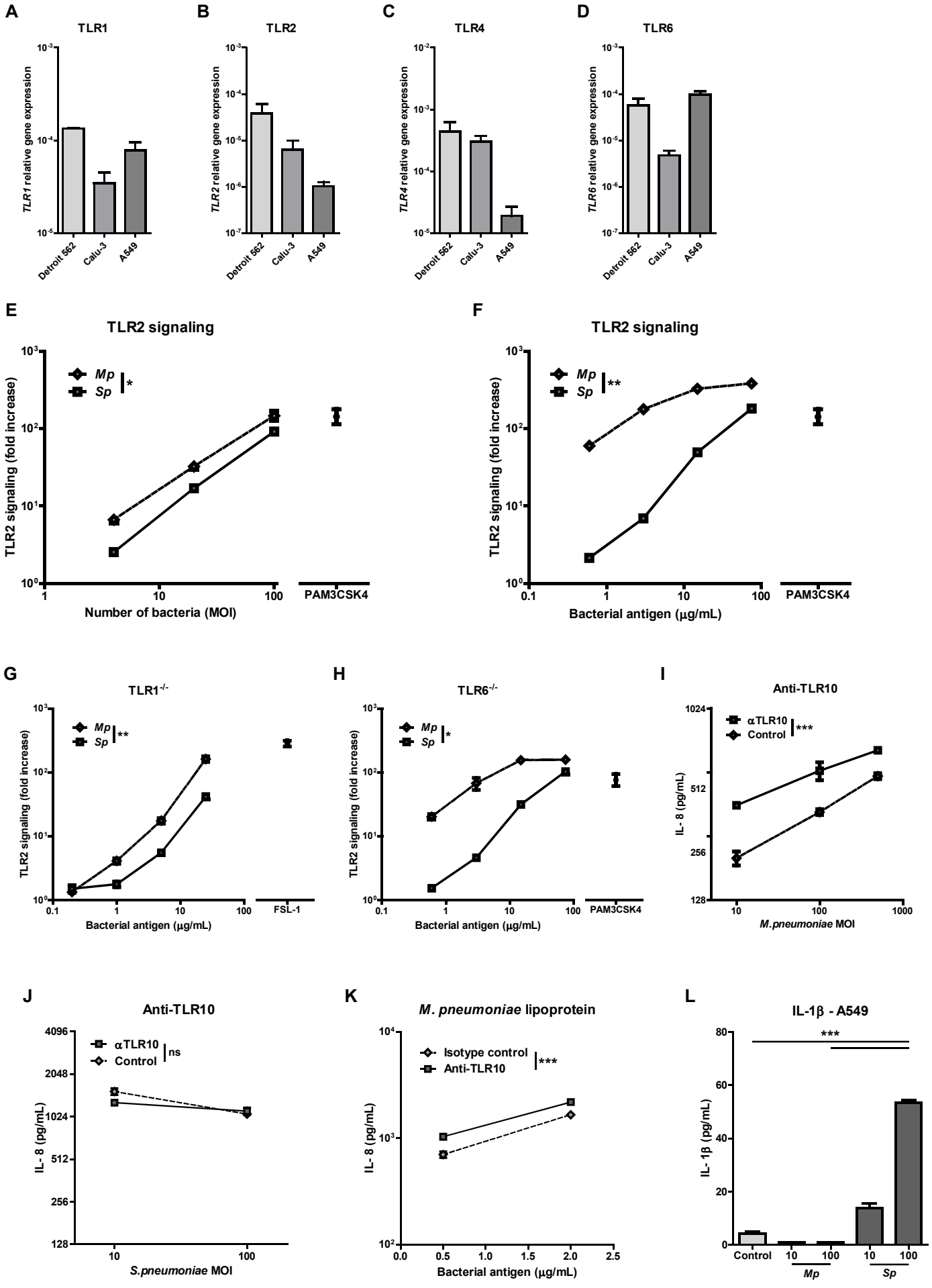
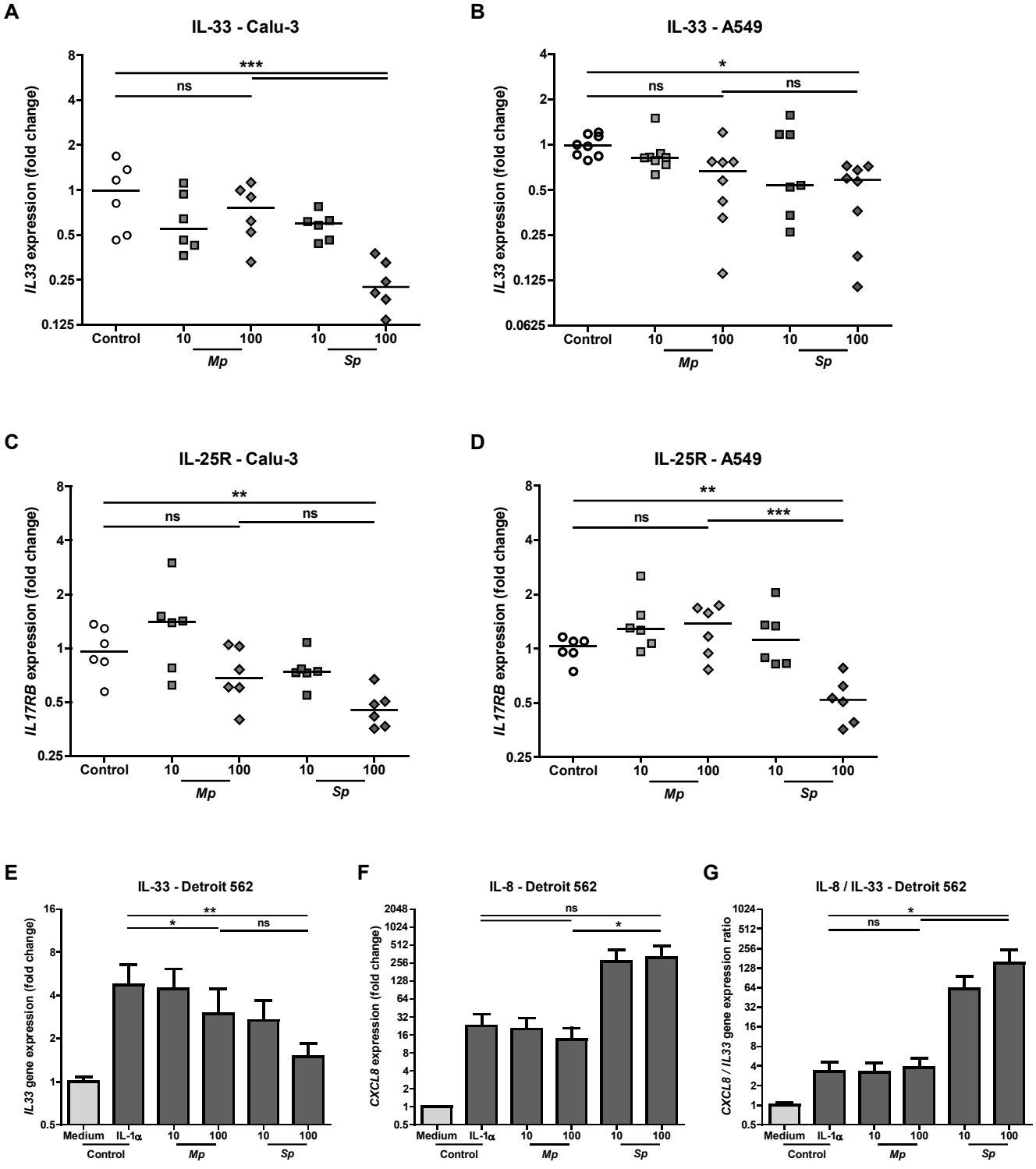


Figure 3. R.C.A. de Groot et al.




A
IL-33 - Calu-3
IL33 expression (fold change)
***
ns
Control
10
100
10
100
Mp
Sp
B
IL-33 - A549
IL33 expression (fold change)
*
ns
ns
C
IL-25R - Calu-3
IL17RB expression (fold change)
**
ns
ns
D
IL-25R - A549
IL17RB expression (fold change)
**
ns
***
E
IL-33 - Detroit 562
IL33 gene expression (fold change)
**
*
ns
Medium
IL-1α
Control
F
IL-8 - Detroit 562
CXCL8 expression (fold change)
ns
*
G
IL-8 / IL-33 - Detroit 562
CXCL8 / IL33 gene expression ratio
*
ns

**Supplementary figure 1**

(A-C) Figure 1 data visualizing variation between donors. Dots represent values of individual donors. Lines connect data points of every individual donor. *p<0.05, **p<0.01 (Repeated measures ANOVA with post-hoc Bonferroni correction).

**Supplementary figure 2**

Respiratory epithelial cells were stimulated with *M. pneumoniae* and *S. pneumoniae* with multiplicity of infection 10 or 100. (A-D) *IL6* and *CXCL8* gene expression was assessed at five hours (n=4-10/group). (E) *CCL20* gene expression was assessed after five hours (n=5-11/group). (F-H) *CCL2* gene expression was assessed after five hours (n=5-12/group). (I) *S. pneumoniae* Colony Forming Units (CFUs) after 0 and 18 hours of incubation with A549 cells. (J) IL-8 levels in culture supernatant after stimulation of A549 cells with live *M. pneumoniae* and *S. pneumoniae* at different MOIs. (A-H) Data shown of at least two independent experiments. (I-J) Data of one representative experiment. Dots represent biological replicates and lines group medians. Bars represent group means and error bars SEM. *p<0.05, **p<0.01, ***p<0.001 (ANOVA with post-hoc Bonferroni correction).

**Supplementary figure 3**

(A-C) Respiratory epithelial cells were stimulated with 100 ng/mL FSL-1 or 1 µg/mL PAM3CSK4. *CCL20* gene expression was assessed after five hours (n=3-10/group). Dots represent biological replicates and lines group medians. ***p<0.001 (ANOVA with post-hoc Bonferroni correction).

(D-E) A549 cells were stimulated with *Haemophilus influenzae* with multiplicity of infection 1, 10 or 100 or 10-100 ng/mL LPS. *IL6* and *CXCL8* gene expression was assessed at five hours (n=2-3/group). (F) TLR4 signaling as assessed by fold increase in bioluminescence of TLR4-luciferase reporter cells upon stimulation with different doses of bacteria (n=3/dose). (G) Mean fold increase in bioluminescence by TLR2 reporter assays after stimulation with alive or heat-killed *M. pneumoniae* or *S. pneumoniae* at MOI 10 (H) IL-6 levels in culture medium after 24 hours of stimulation of A549 cells with *M. pneumoniae* in the presence of TLR10 blocking antibody or isotype control. (I) IL-6 levels in culture medium after 24 hours of stimulation with 2 μg/mL of *M. pneumoniae* lipoproteins with either a TLR10-blocking antibody or isotype control. (L) IL-1β levels in culture medium after 24 hours of stimulation with live *M. pneumoniae* or *S. pneumoniae* at MOI 10 or 100. (A-C) Data shown of at least two independent experiments. (D-G) Data shown of one experiment. (A-E) Dots represent biological replicates and lines group medians. Bars represent group means and error bars SEM. *p<0.05, **p<0.01, ***p<0.001 (ANOVA with post-hoc Bonferroni correction or comparing $EC_{50}$ of fitted dose-response curves).

**Supplementary figure 4**

(A-C) Detroit 562 cells were stimulated with 100 ng/mL FSL-1 or vehicle controls and simultaneous with 5 ng/mL IL-1 alpha or vehicle control. *IL33* and *CXCL8* gene expression was assessed after 5 hours. Bars represent group means and error bars SEM. Data of 2 independent experiments.

# Supplemental Information

## Full statistical report

All performed tests are reported in the tables below with exact p-values. Technical replicates were averaged and statistical tests were applied on biological replicates or individual patients. We assumed log-normal distribution for cytokine levels, gene expression ratios and TLR signaling data.

### Figure 1

Analyses were paired for individual donors.

| | **Data sets** | **Statistical test** | **Exact p-value** |
|---|---|---|---|
| **1A** | All groups | Repeated measures ANOVA | .0112 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 1.406 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 4.462 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 3.057 |
| **1B** | All groups | Repeated measures ANOVA | .0093 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 1.652 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 4.307 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 2.655 |
| **1C** | All groups | Repeated measures ANOVA | .0132 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 2.803 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 4.543 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 1.740 |

**Figure 2**

| | Data sets | Statistical test | Exact p-value |
|---|---|---|---|
| **2A** | All groups | One-way ANOVA | < .0001 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 1.153 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 6.546 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 5.394 |
| **2B** | All groups | One-way ANOVA | < .0001 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 1.748 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 5.852 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 4.104 |
| **2C** | All groups | One-way ANOVA | .0005 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = .0437 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 4.618 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 4.574 |
| **2D** | All groups | One-way ANOVA | .0008 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 1.425 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 4.491 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 3.066 |
| **2E** | All groups | One-way ANOVA | < .0001 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 4.671 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 10.48 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 5.395 |
| **2F** | All groups | One-way ANOVA | < .0001 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 3.023 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 8.874 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 4.856 |

**Figure 3**

Both doses and fold increases in TLR2 signaling were $^{10}$log-transformed. A three parameter dose-response curve with constrained top and bottom were fitted to the data. All dose-response curves were assumed to have a standard slope. We tested if log($EC_{50}$) parameters were the same or different for *M. pneumoniae* and *S. pneumoniae*.

| | **Data sets** | **Statistical test** | **Exact p-value** |
|---|---|---|---|
| **3E** | All groups | Log($EC_{50}$) same for both data sets | < .0001 |
| | | F(DFn, DFd) | 52.46 (1,14) |
| | *Mp* (all doses) | *Mp* $EC_{50}$ | 9.942 |
| | *Sp* (all doses) | *Sp* $EC_{50}$ | 20.47 |
| | *Mp* and *Sp* | $EC_{50}$ ratio | 2.059 |
| **3F** | All groups | Log($EC_{50}$) same for both data sets | < .0001 |
| | | F(DFn, DFd) | 2654 (1,20) |
| | *Mp* (all doses) | *Mp* $EC_{50}$ | .2998 |
| | *Sp* (all doses) | *Sp* $EC_{50}$ | 8.355 |
| | *Mp* and *Sp* | $EC_{50}$ ratio | 27.87 |
| **3G** | All groups | Log($EC_{50}$) same for both data sets | < .0001 |
| | | F(DFn, DFd) | 126.4 (1,20) |
| | *Mp* (all doses) | *Mp* $EC_{50}$ | 5.750 |
| | *Sp* (all doses) | *Sp* $EC_{50}$ | 16.74 |
| | *Mp* and *Sp* | $EC_{50}$ ratio | 2.911 |
| **3H** | All groups | Log($EC_{50}$) same for both data sets | < .0001 |
| | | F(DFn, DFd) | 761.7 (1,20) |
| | *Mp* (all doses) | *Mp* $EC_{50}$ | .4562 |
| | *Sp* (all doses) | *Sp* $EC_{50}$ | 7.251 |
| | *Mp* and *Sp* | $EC_{50}$ ratio | 15.89 |

**Figure 4**

| | Data sets | Statistical test | Exact p-value |
|---|---|---|---|
| **4A** | All groups | One-way ANOVA | .0001 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 1.061 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 5.538 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 4.477 |
| **4B** | All groups | One-way ANOVA | .0219 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 2.299 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 3.177 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = .8780 |
| **4C** | All groups | One-way ANOVA | .0008 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 1.501 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 3.578 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 2.077 |
| **4D** | All groups | One-way ANOVA | < .0001 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 1.318 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 3.710 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 5.028 |
| **4E** | All groups | One-way ANOVA | .0038 |
| | IL-1α control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 2.860 |
| | IL-1α control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 4.235 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 1.375 |
| **4F** | All groups | One-way ANOVA | .0426 |
| | IL-1α control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = .4484 |
| | IL-1α control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 2.512 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 2.960 |
| **4G** | All groups | One-way ANOVA | .0214 |
| | IL-1α control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = .4423 |
| | IL-1α control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 3.256 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 2.814 |

**Supplementary Figure 1**

Statistical tests are the same as for Figure 1.

**Supplementary Figure 2**

| | Data sets | Statistical test | Exact p-value |
|---|---|---|---|
| **S2A** | All groups | One-way ANOVA | < .0001 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 4.390 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 9.925 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 5.053 |
| **S2B** | All groups | One-way ANOVA | < .0001 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = .4903 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 6.971 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 5.796 |
| **S2C** | All groups | One-way ANOVA | < .0001 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 3.445 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 11.79 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 7.230 |
| **S2D** | All groups | One-way ANOVA | < .0001 |
| | Control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 6.448 |
| | Control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 7.510 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = .5439 |
| **S2E** | All groups | One-way ANOVA | < .0001 |
| | IL-1α control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 5.154 |
| | IL-1α control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 5.724 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = .5295 |
| **S2F** | All groups | One-way ANOVA | .0685 |
| | IL-1α control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | n/a |
| | IL-1α control vs *Sp* 100 | Post-hoc Bonferroni's MCT | n/a |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | n/a |
| **S2G** | All groups | One-way ANOVA | .0008 |
| | IL-1α control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 3.585 |
| | IL-1α control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 3.718 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = .1239 |
| **S2H** | All groups | One-way ANOVA | < .0001 |
| | IL-1α control vs. *Mp* 100 | Post-hoc Bonferroni's MCT | t = 2.004 |
| | IL-1α control vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 5.315 |
| | *Mp* 100 vs *Sp* 100 | Post-hoc Bonferroni's MCT | t = 2.904 |

**Supplementary Figure 3**

| | Data sets | Statistical test | Exact p-value |
|---|---|---|---|
| **S3A** | All groups | One-way ANOVA | < .0001 |
| | Control vs. FSL-1 | Post-hoc Bonferroni's MCT | t = 21.02 |
| | Control vs PAM3CSK4 | Post-hoc Bonferroni's MCT | t = 12.28 |
| **S3B** | All groups | One-way ANOVA | < .0001 |
| | Control vs. FSL-1 | Post-hoc Bonferroni's MCT | t = 27.65 |
| | Control vs PAM3CSK4 | Post-hoc Bonferroni's MCT | t = 13.74 |
| **S3C** | All groups | One-way ANOVA | < .0001 |
| | Control vs. FSL-1 | Post-hoc Bonferroni's MCT | t = 11.10 |
| | Control vs PAM3CSK4 | Post-hoc Bonferroni's MCT | t = 10.53 |

**Supplementary Figure 4**

| | Data sets | Statistical test | Exact p-value |
|---|---|---|---|
| **S4A** | IL-1α vs. IL-1α + FSL-1 | Paired T-test | .0244 |
| **S4B** | IL-1α vs. IL-1α + FSL-1 | Paired T-test | .2395 |
| **S4C** | IL-1α vs. IL-1α + FSL-1 | Paired T-test | .6961 |

**Detailed methods for quantitative PCR**

Epithelial cell samples were washed with PBS and dry cells were flash frozen until mRNA extraction. Epithelial cells were lysed in culture plate using lysis buffer from the Nucleospin RNA extraction kit (Macherey-Nagel, Düren, Germany). RNA was extracted according to manufacturer's instructions and then incubated with DNAse I (Promega, Madison, USA) for 15 minutes at room temperature. Extracted RNA was quantified with a DS-11 FX spectrophotometer (DeNovix, Wilmington, USA) and $A_{260}/A_{280}$ ratios were used to assess RNA purity. 1 µg of RNA was used for reverse transcription using the Sensifast cDNA synthesis kit according to manufacturer's instructions (Bioline Reagents, London, UK). qPCR target sequences for forward and reverse primers were on different exons separated by introns larger than 500 base pairs to avoid amplification of contaminant genomic DNA. Primer specificity was tested *in silico* using BLAST and *in vitro* verification of specificity was performed with melting curve analysis and gel electrophoresis. Only primer sets with PCR efficiencies between 95-105% were accepted. The following primer sets were used: *GAPDH*: Fw 5'-GTCGGAGTCAACGGATT-3', Rv 5'-AAGCTTCCCGTTCTCAG-3', *CCL2* (MCP-1): Fw 5'-TCCAGCATGAAAGTCTCTG-3', Rv 5'-CGAGCCTCTGCACTGA-3', *CCL20* (MIP-3α): Fw 5'-GAAGGCTGTGACATCAATG-3', Rv 5'-CCCCAGCAAGGTTCTT-3', *IL33*: Fw 5'-AACACCCCTCAAATGAATC-3', Rv 5'-CTTGCATTCAAATGAAACAC-3', *CXCL8* (IL-8): Fw 5'-CCGGAAGGAACCATCT-3', Rv 5'-TTGGGGTGGAAAGGTT-3', *IL17RB* (IL-25 receptor): Fw 5'-GGCACGAAAGGATCAAG-3', Rv 5'-CTGCAATGGTTTTGAAGAA-3'. The reaction mix consisted of SensiMix SYBR & Fluorescein Kit (Bioline reagents), 10 µM of forward and reverse primer, $Mg^{2+}$ final concentrations were 4.0 mM and total reaction volume was 20 µL. PCR reactions were performed in clear Hard-Shell PCR plates (Bio-Rad Laboratories, Hercules, USA) sealed with Microseal B adhesive seals (Bio-Rad Laboratories, Hercules, USA) in a CFX96 Real-Time system C1000 (Bio-Rad laboratories). The PCR protocol consisted of 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C

followed by  at Amplification and melting curves were inspected visually and technical replicates were considered adequate if they differed no more than 1 cycle. The quantification cycle ($C_q$) was determined using Bio-rad CFX manager algorithm (Bio-Rad Laboratories). *GAPDH* was used as a reference gene for normalizing gene expression. Relative gene expression was normalized to medium controls and expressed as a ratio.