

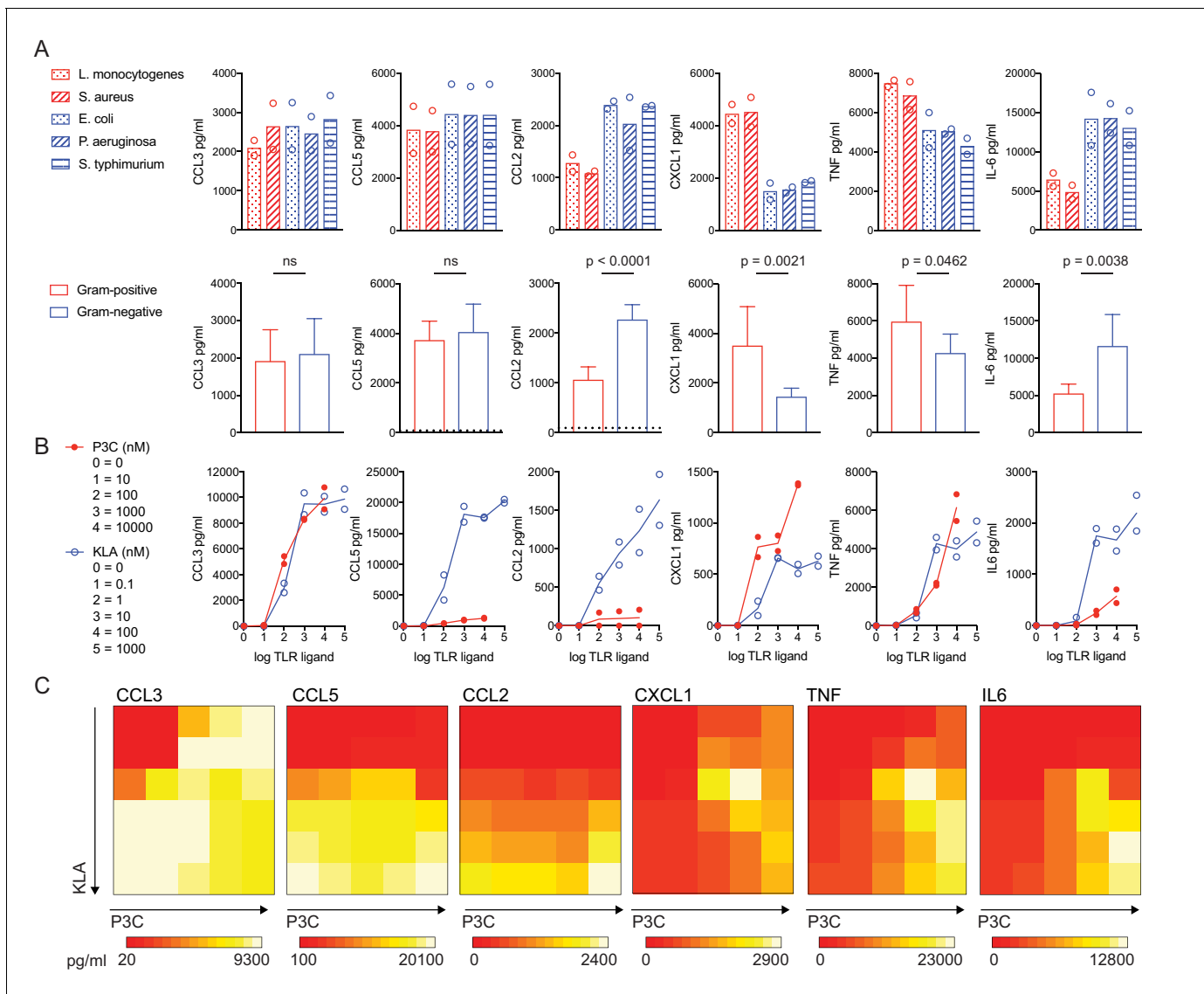


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## Figures and figure supplements

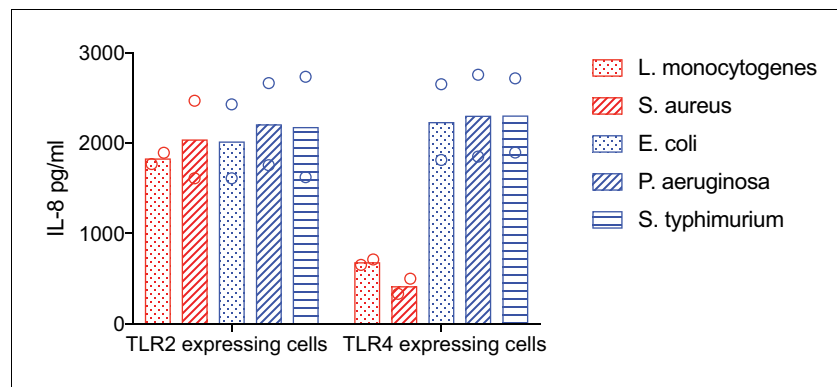
IFN-mediated negative feedback supports bacteria class-specific macrophage inflammatory responses

**Rachel A Gottschalk et al**



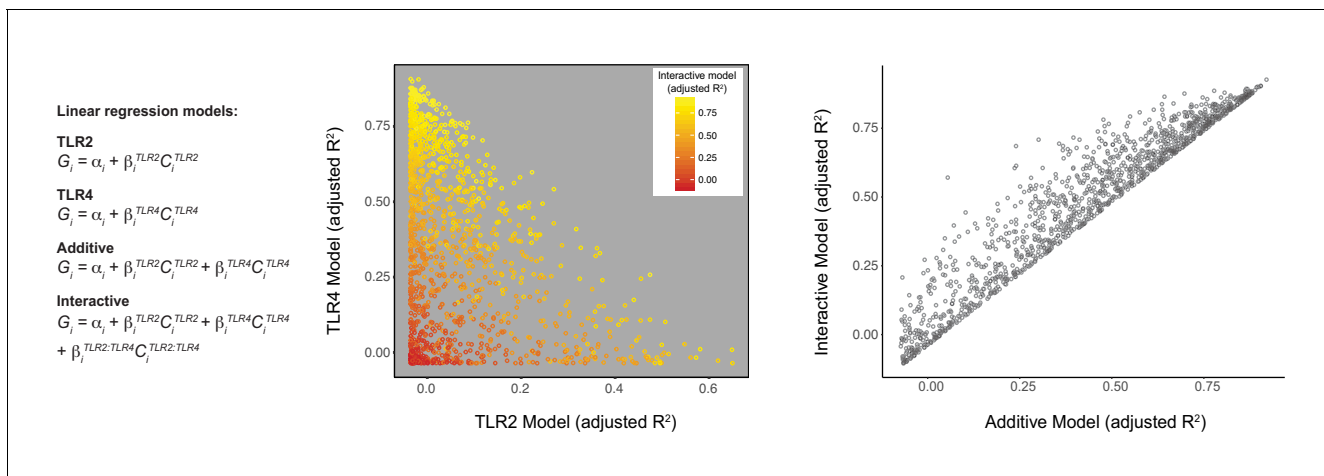
**Figure 1.** TLR pathway non-additivity supports context-specific inflammatory responses. BMDM were stimulated with heat-killed bacteria at an MOI of 100 (Red = Gram-positive: *L. monocytogenes* or *S. aureus*; Blue = Gram-negative: *E. coli*, *P. aeruginosa*, or *S. Typhimurium*) or soluble TLR ligand (P3C = TLR2 ligand Pam3CKS4; KLA = TLR4 ligand KDO2-LipidA), as indicated. After 12 hr (A) or 6 hr (B, C) supernatants were collected and cytokines were quantified using cytometric bead array. Data points represent independent experiments. (A) Data from bacterial species (upper row) were combined from three independent experiments to show the distinct effects of Gram-positive and Gram-negative bacteria on inflammatory cytokine production in BMDM (lower row); statistical significance based on unpaired t-tests, between Gram-negative- and Gram-positive-stimulated samples. Dotted lines in the lower row represent the mean values for unstimulated BMDM (0–12 hr). In some cases, these controls are not visible because they are below the level of detection (a zero value). Ligand concentrations in (C) correspond to single ligand concentrations shown in (B), in pairwise combinations. Data are representative of three independent experiments.

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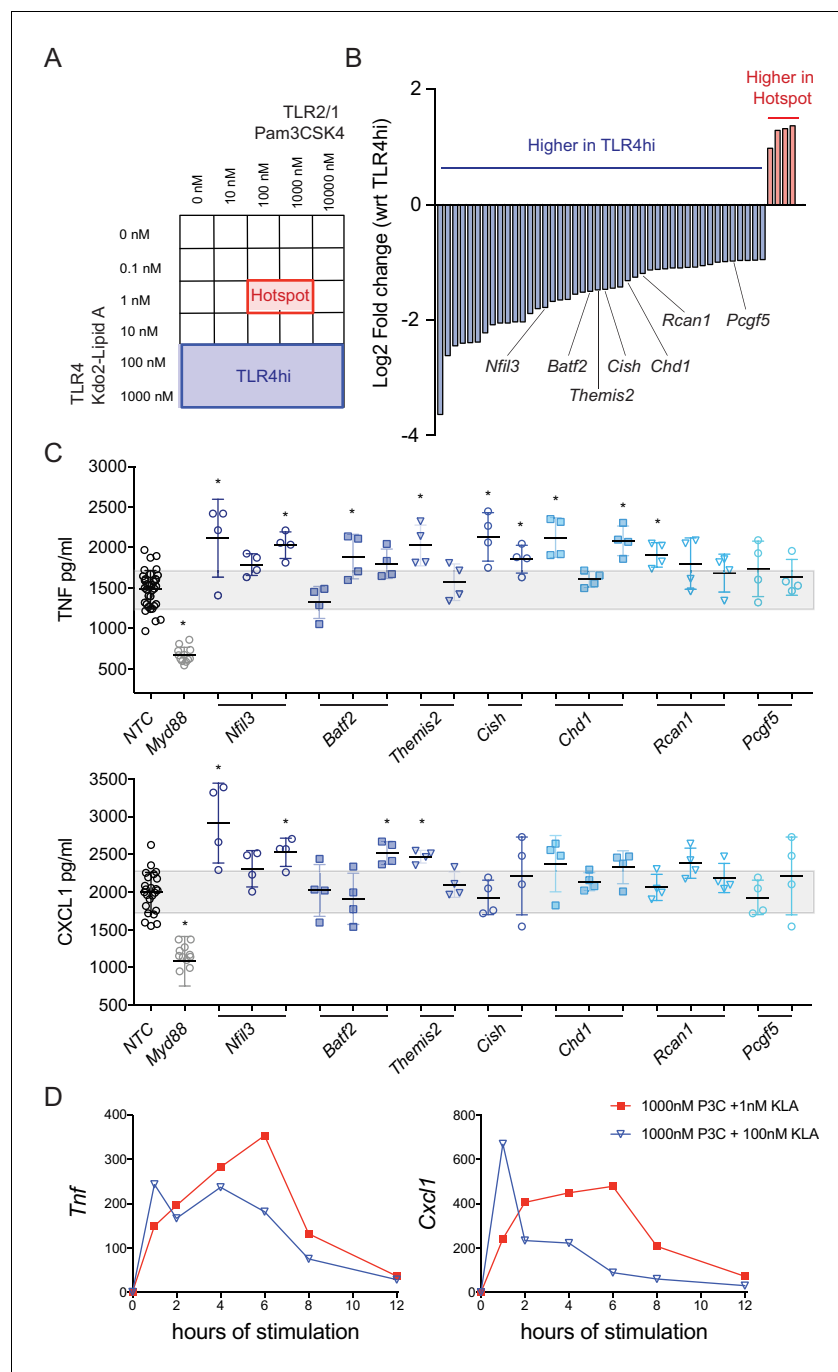
**Figure 1—figure supplement 1.** TLR2 and TLR4 ligation by heat-killed bacteria. HEK293 cells stably expressing either TLR2 or TLR4 were stimulated with the indicated heat-killed bacteria for 20 hr. Supernatants were analyzed by ELISA and IL-8 production was used as a readout of TLR ligation and downstream function. IL-8 was not detected in unstimulated HEK293. Data are pooled from two independent experiments.

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**Figure 1—figure supplement 2.** Linear regression analysis highlights non-additive TLR pathway interactions. Using the titration matrix conditions shown in **Figure 1**, BMDM were stimulated for 2 hr, induced gene expression was measured by microarray, and linear regression analysis was performed for each gene across the 30-condition dataset. Points represent individual gene probes induced above 1.5-fold in at least three conditions.

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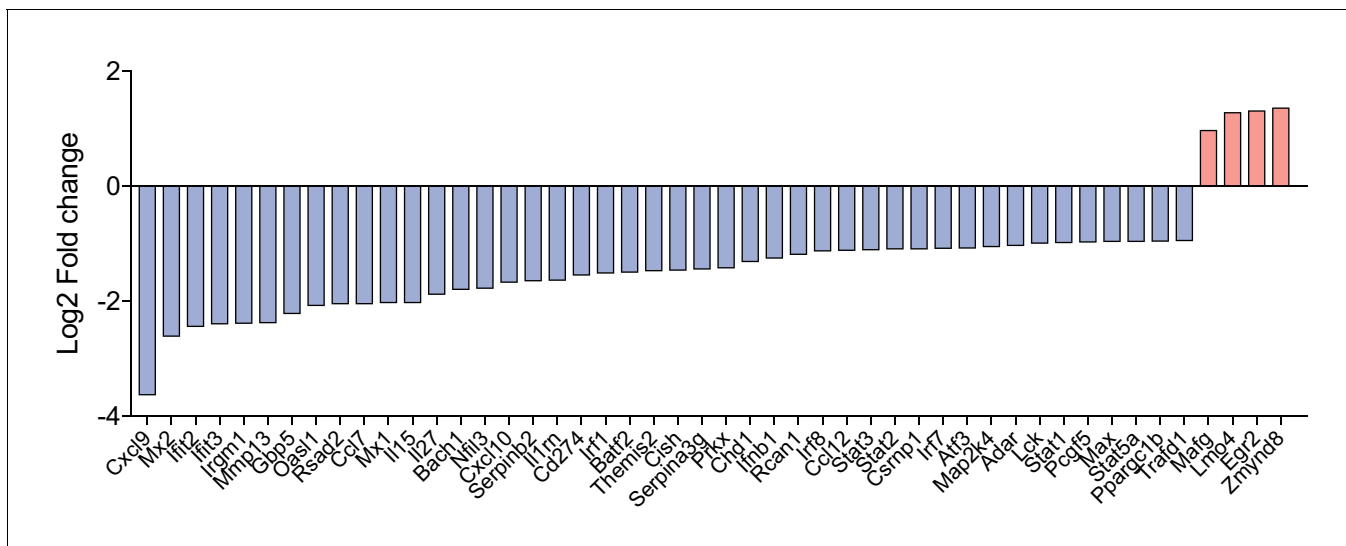
**Figure 2.** Regulator genes are induced above a threshold of TLR4 ligand. (A, B) Using the titration matrix conditions shown in **Figure 1**, BMDM were stimulated for 2 hr and induced gene expression was measured by microarray. Differential gene expression analysis was performed between the hotspot and TLR4 high regions of the dual ligand titration matrix. Candidate regulator genes preferentially induced by high TLR4 ligand are noted (B). (C) BMDM were treated with three independent siRNA per candidate regulator (siRNA resulting in less than 50% viability were excluded), MyD88 siRNA, or non-targeting control (NTC) siRNA for 48 hr prior to stimulation with 1000 nM P3C and 100 nM KLA (TLR4hi condition). After 6 hr of stimulation, TNF or CXCL1 was quantified in supernatants by ELISA and values were normalized based on cell viability. Individual data points represent wells treated independently with siRNA and TLR ligand, and the gray bar depicts NTC standard deviation. Candidate genes were differentially expressed in three independent microarray or qPCR experiments and siRNA results are representative of two independent experiments. Stars represent statistical significance based on ordinary one-way ANOVA, versus control (\* $p \leq 0.05$ ). (D) BMDM were stimulated with the indicated concentrations of TLR ligand and

Figure 2 continued on next page

*Figure 2 continued*

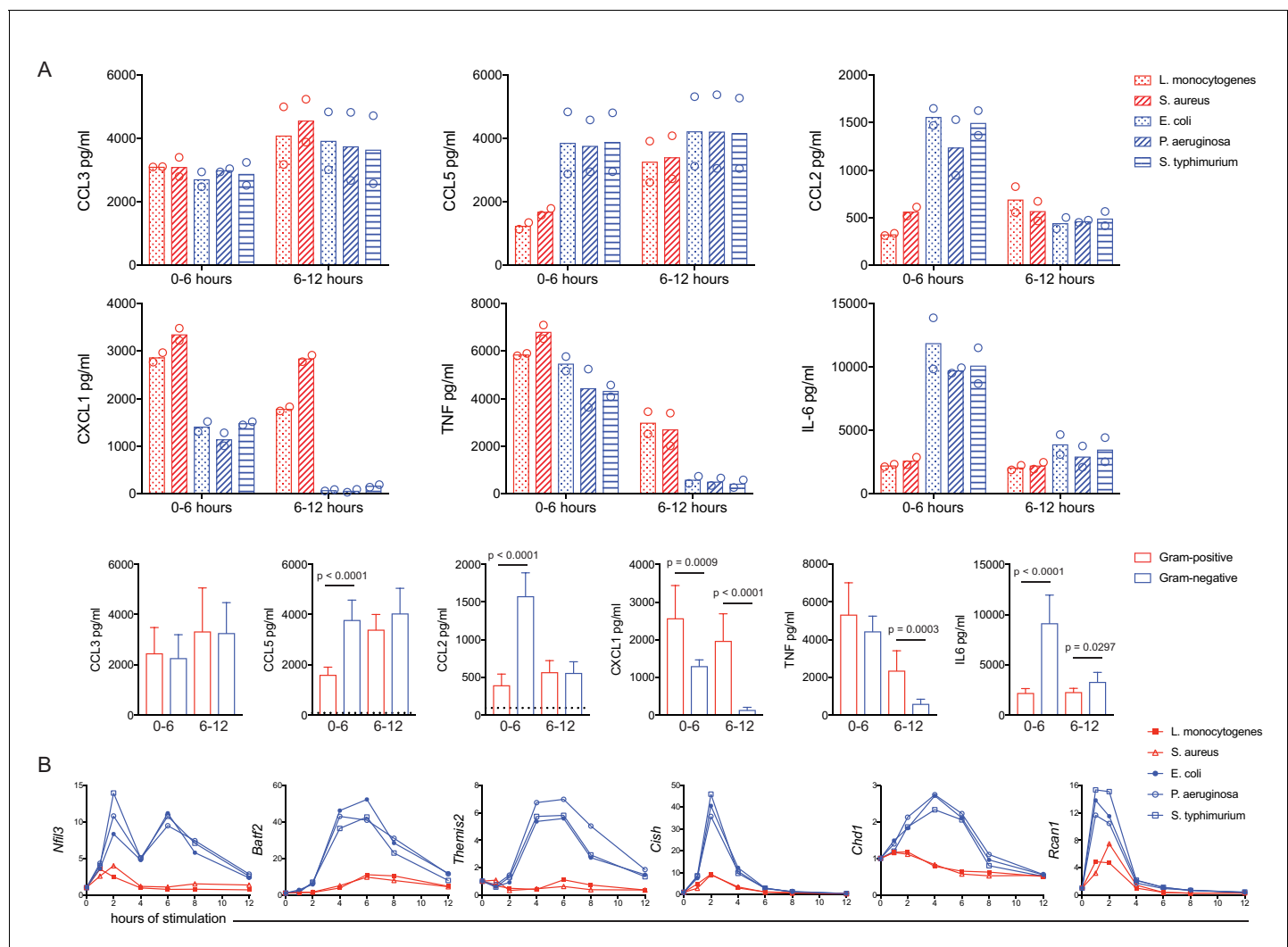
harvested at the indicated time. Gene expression was quantified using qPCR and fold change was calculated compared to unstimulated BMDM. Data are representative of three independent experiments.

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**Figure 2—figure supplement 1.** Genes induced above a threshold of TLR4 activation. Microarray data from **Figure 2B** are expanded so as to show the identities of the TLR4-driven genes (blue) as well as those associated with the titration matrix 'hotspot' (red).

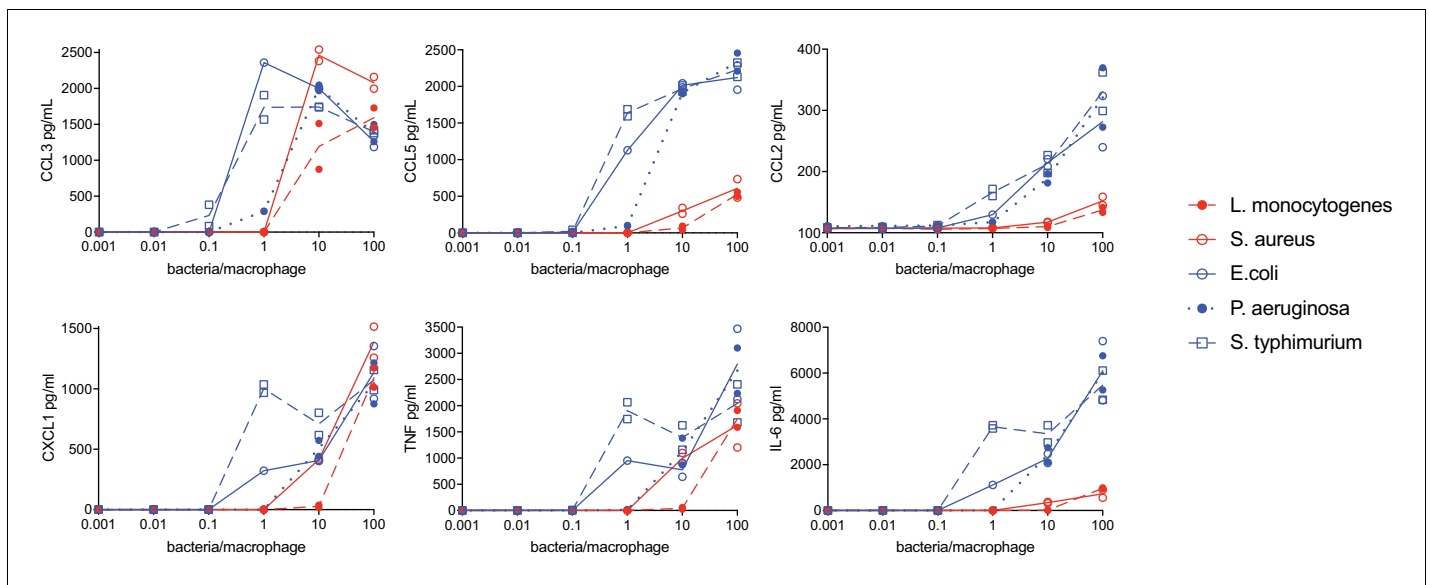
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**Figure 3.** A dual TLR titration matrix predicts bacteria-specific cytokine shutdown and regulator induction. BMDM were stimulated with heat-killed bacteria. (A) After 6 hr of stimulation, supernatants were removed (0–6 hr) and replaced with fresh medium for a subsequent 6 hr of stimulation (6–12 hr). Cytokines were quantified using cytometric bead array and data points are experimental replicates. Data from bacterial species (upper rows) were combined from three independent experiments (lower row) and statistical significance was determined using unpaired t-tests between Gram-negative- and Gram-positive-stimulated samples; where no p-value is indicated, results were not significant. Dotted lines in the lower row represent the mean values for unstimulated BMDM (0–12 hr). In some cases, these controls are not visible because they are below the level of detection (a zero value). (B) Cells were harvested at the indicated time, gene expression was quantified using qPCR, and fold change was calculated compared to unstimulated BMDM. Results are representative of four or more independent experiments.

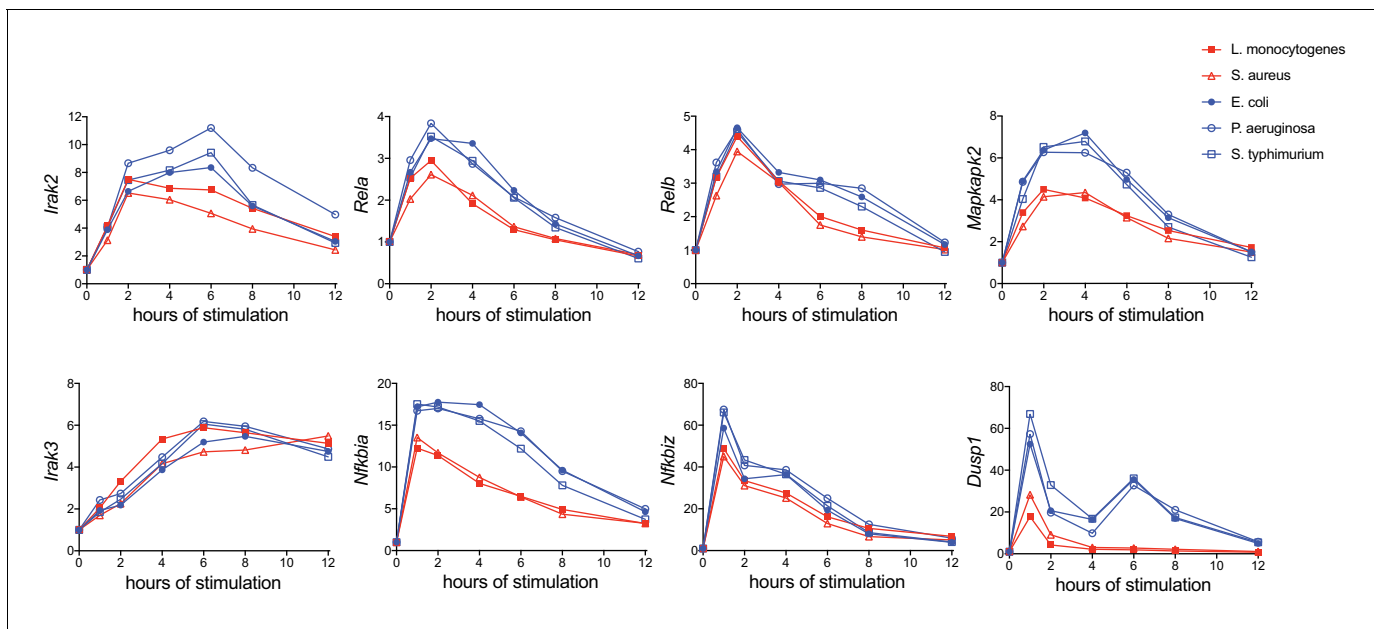
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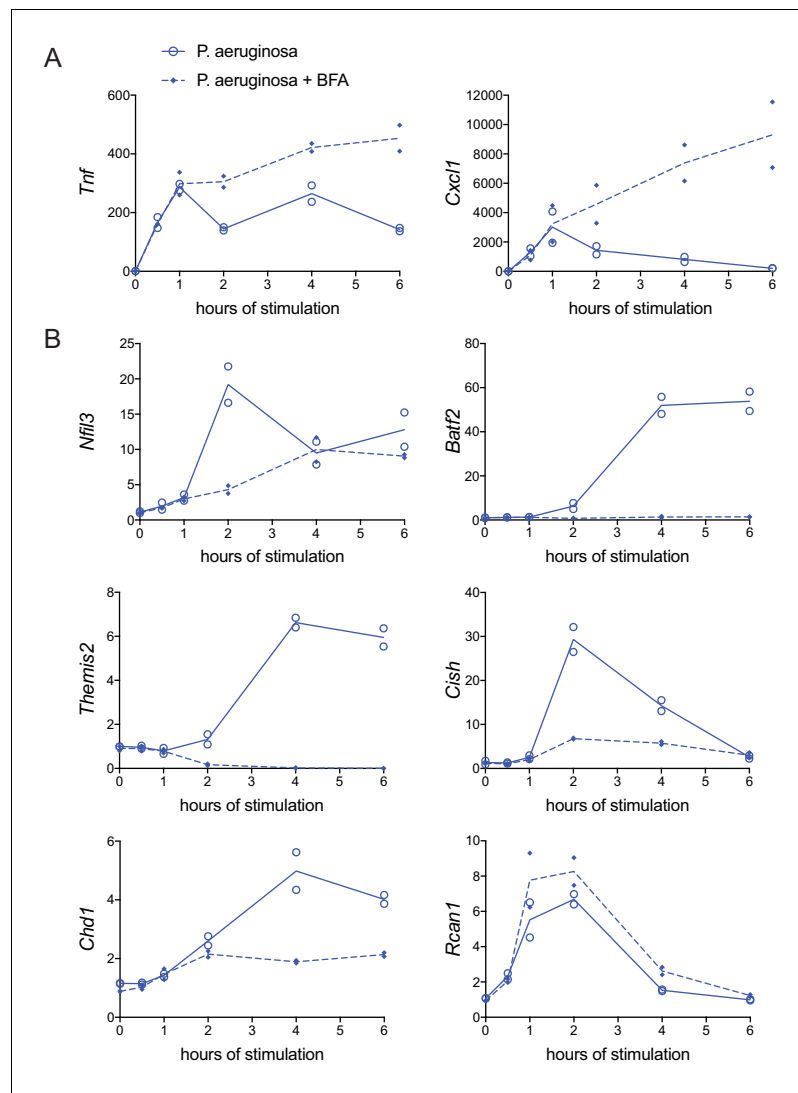


**Figure 3—figure supplement 1.** Macrophage cytokine secretion in response to varying concentrations of heat-killed bacteria. BMDM were stimulated with heat-killed bacteria. After 6 hr of stimulation, supernatants were collected and cytokines were quantified using cytometric bead array. Data points represent replicate wells.

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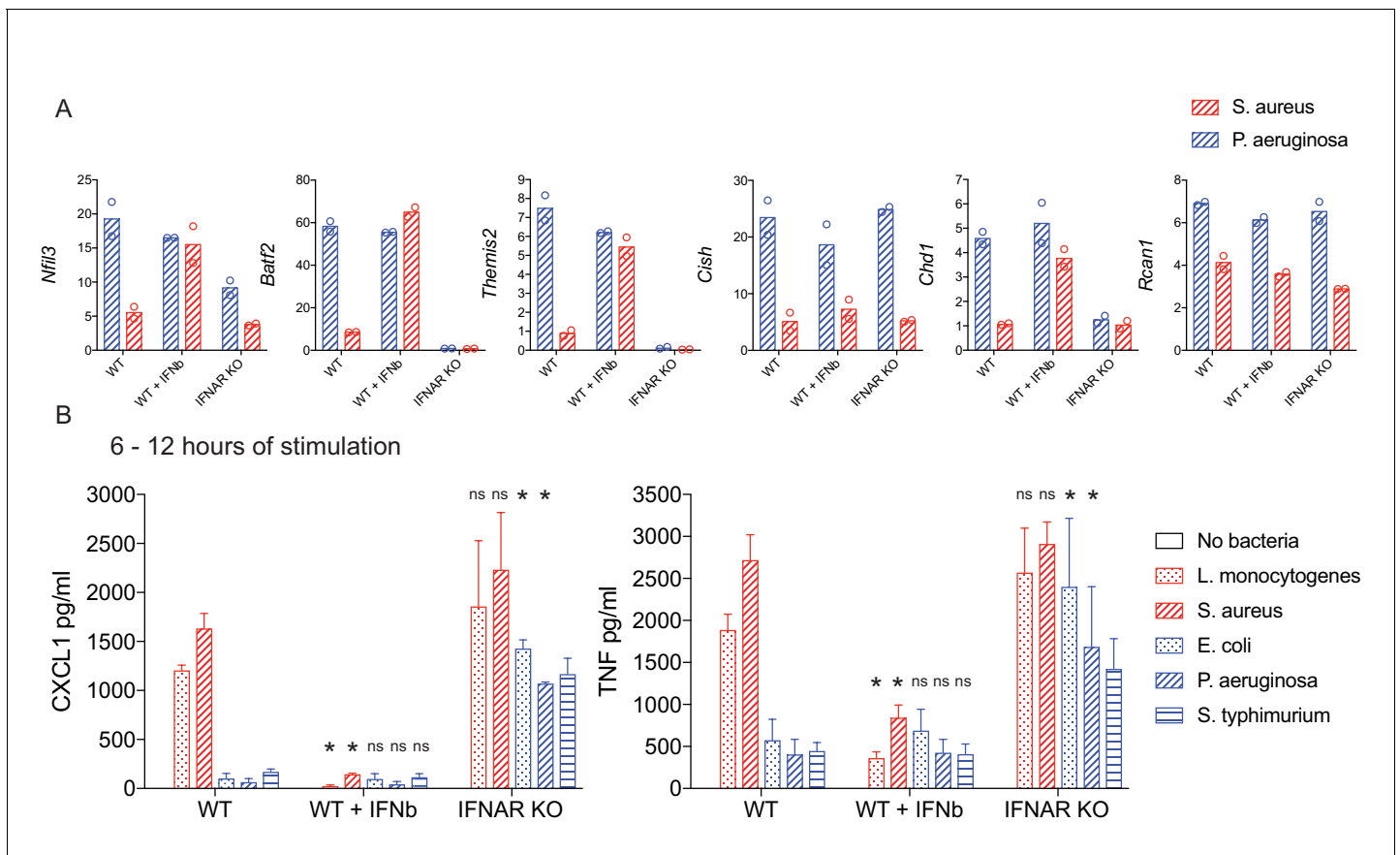


**Figure 3—figure supplement 2.** Known negative regulators of inflammatory signaling are more highly induced by Gram-negative pathogens. Cells were stimulated as in **Figure 3** and mRNA transcripts of known immune regulators that were not identified in our microarray were measured by qPCR. Gene expression was quantified based on fold-change compared to unstimulated BMDM. Results are representative of two independent experiments. DOI: <https://doi.org/10.7554/eLife.46836.010>



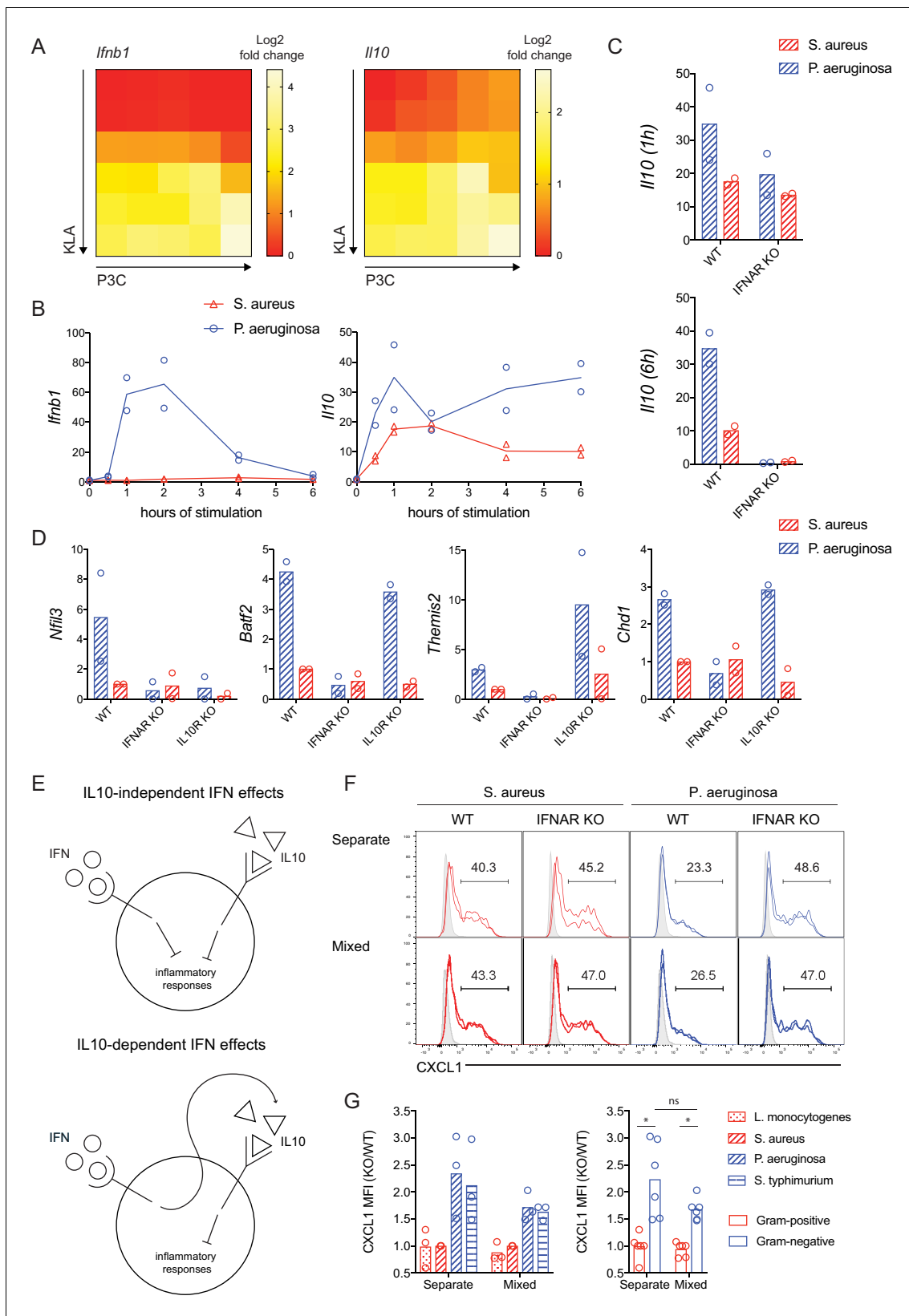
**Figure 4.** Rapid Gram-negative-driven inflammatory response decay and regulator induction are dependent on cytokine feedback. BMDM were stimulated with heat-killed *P. aeruginosa* with or without protein transport inhibitor brefeldin A (BFA). Inflammatory cytokine genes (A) and candidate regulator genes (B) were quantified by qPCR and fold change was calculated compared to unstimulated BMDM. Data shown are from two independent experiments.

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**Figure 5.** IFN-mediated negative feedback supports bacteria class-specific responses. WT or IFNAR KO BMDM were stimulated with heat-killed bacteria, with the addition of IFN- $\beta$  after one hour of stimulation, where indicated. **(A)** Induced gene expression was measured by qPCR and fold change was calculated compared to unstimulated BMDM. Data are shown for the timepoint of maximum WT expression over a 6 hr time course: 2 hr *Nfil3*, 4 hr *Batf2*, 4 hr *Themis2*, 2 hr *Cish*, 4 hr *Chd1*, and 2 hr *Rcan1*. Data shown are from two independent experiments. **(B)** After 6 hr of stimulation, supernatants were removed and replaced with fresh medium for a subsequent 6 hr of stimulation. The 6–12 hr supernatants were analyzed by cytometric bead array. Cytokine data are pooled from four independent experiments ( $n = 4$ ,  $n = 3$ , and  $n = 3$  for WT, WT + IFN- $\beta$ , and IFNAR KO, respectively). Stars represent statistical significance based on ordinary two-way ANOVA, comparing WT + IFN- $\beta$  or IFNAR KO treatment groups to WT for each respective bacterial stimulation ( $*p \leq 0.05$ ).

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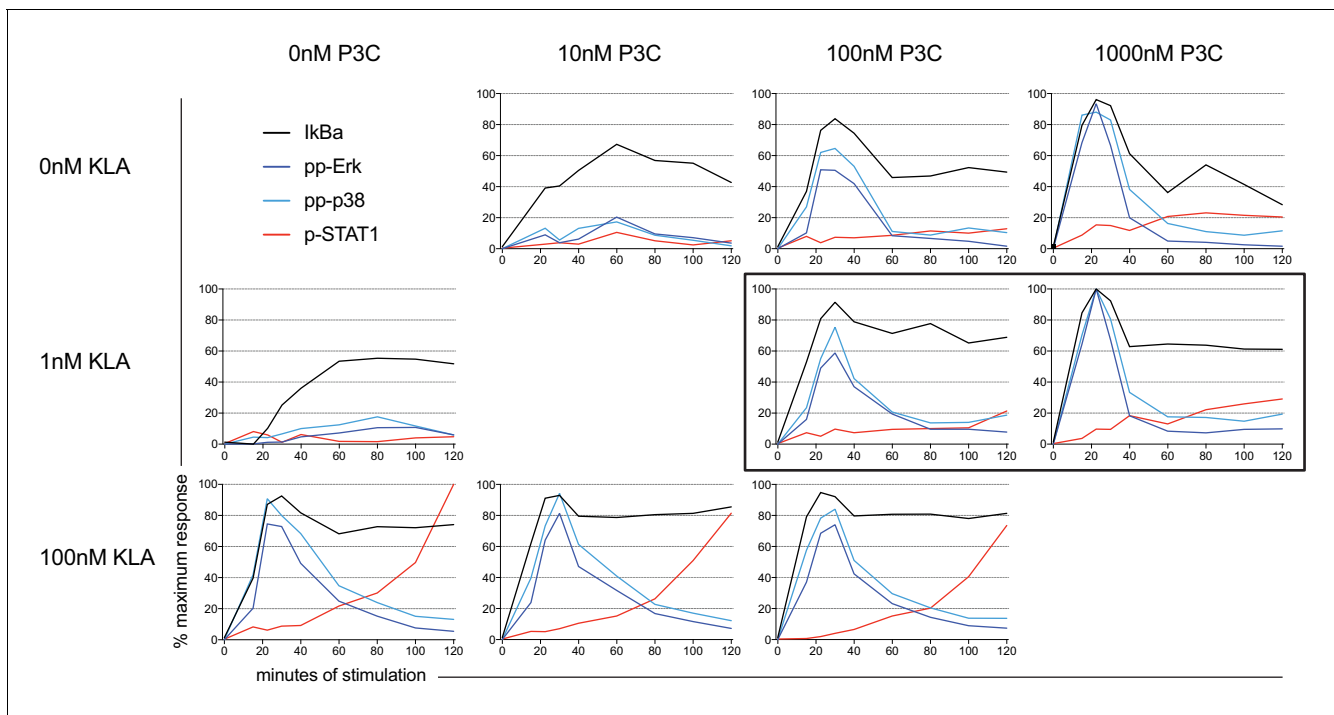


**Figure 6.** IFN regulates inflammatory responses independent of IL-10 production. (A) TLR-induced expression of *Ifnb1* and *Il10* at 2 hr, analyzed from microarray data described in **Figure 1**. (B–D) WT, IFNAR KO, or IL10R KO BMDM were stimulated with heat-killed *S. aureus* or *P. aeruginosa*, gene expression was measured by qPCR, and fold change was calculated compared to unstimulated BMDM. Data shown represent experimental replicates. Figure 6 continued on next page

## Figure 6 continued

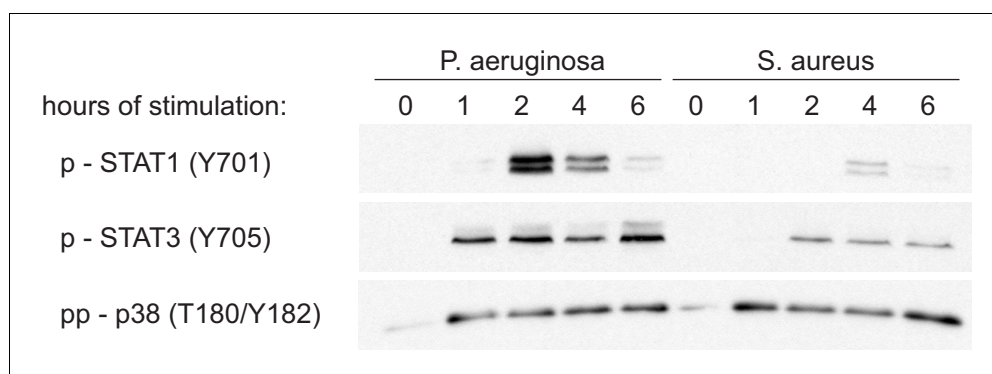
In D, the time of maximum expression is plotted (2 hr *Nfil3*, 4 hr *Batf2*, 4 hr *Themis2*, and 4 hr *Chd1*) and the experimental replicates were normalized with respect to WT *S. aureus*. (E) Models of IFN and IL10 mediated anti-inflammatory function. (F, G). WT (CD45.1+) and IFNAR KO (CD45.1-) BMDM were stimulated with the indicated heat-killed bacteria, separately or in mixed BMDM cultures (1:1 of WT:KO). BFA was added for the final 3 hr of an 8 hr stimulation. (F) Representative flow cytometry histograms from replicate wells are shown with average frequencies. Shaded histograms represent unstimulated BMDM of the indicated genotype and culture type. (G) CXCL1 quantification as a KO/WT ratio was normalized to WT cells stimulated with *S. aureus* and pooled from three independent experiments. The same data are shown for individual bacterial species or pooled between bacterial class, with stars representing statistical significance based on ordinary one-way ANOVA (\* $p \leq 0.05$ ).

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**Figure 6—figure supplement 1.** Signaling kinetics of dual-ligand stimulation. BMDM were stimulated with varying concentrations of KLA and P3C. Phospho-flow showing phosphorylation of Erk, p38, and STAT1, or degradation of IkB $\alpha$ . STAT1 phosphorylation is only seen at high KLA concentrations (lower panels), while other molecules are activated by multiple doses of KLA and/or P3C. Hotspot conditions are highlighted by the black rectangle. Data shown as % of maximum response.

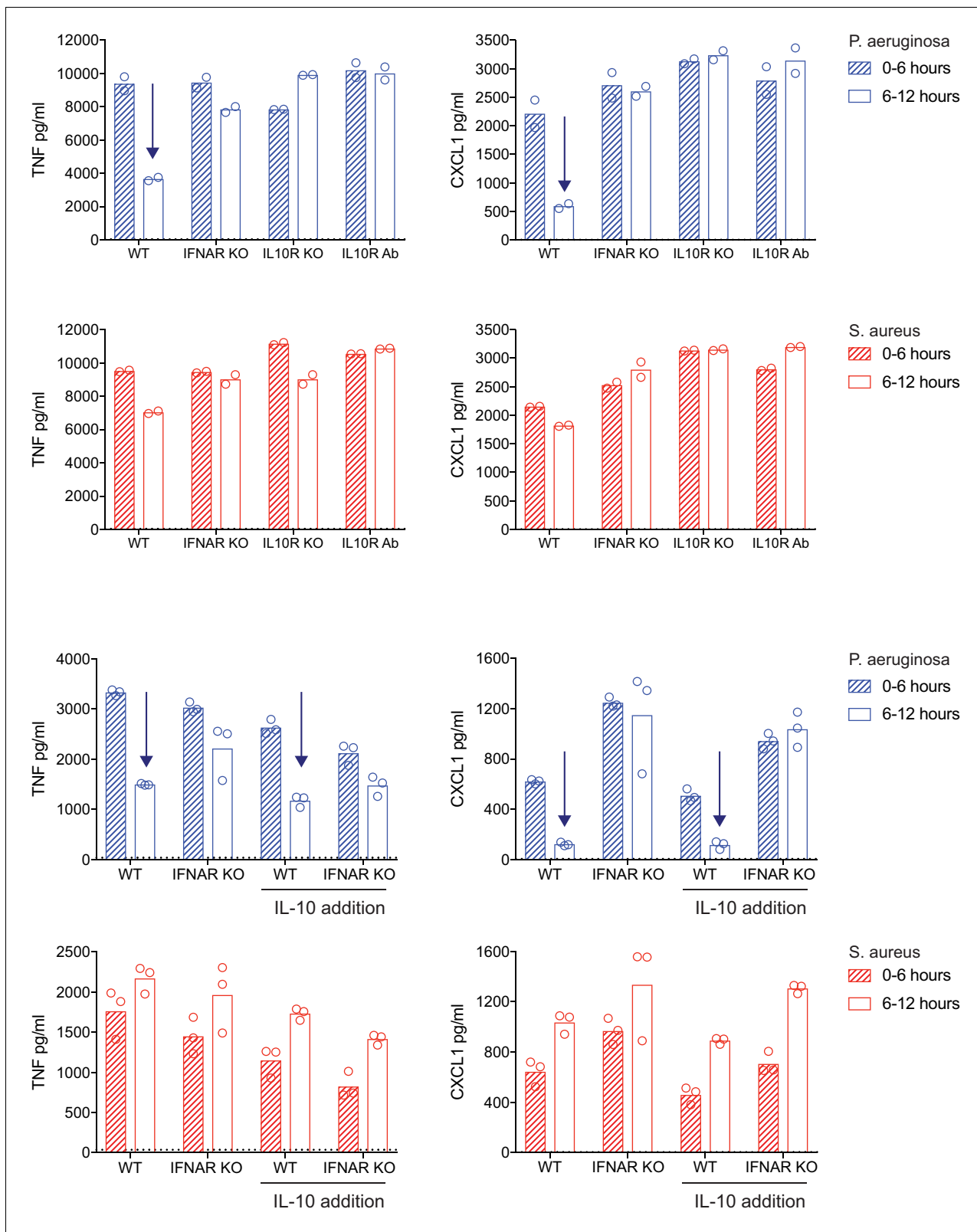
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**Figure 6—figure supplement 2.** STAT activation upon stimulation with heat-killed bacteria. Western blots show strong phosphorylation of STAT1 and STAT3 upon stimulation with *P. aeruginosa* but not *S. aureus*, especially at early timepoints. Blots are representative of three experiments.

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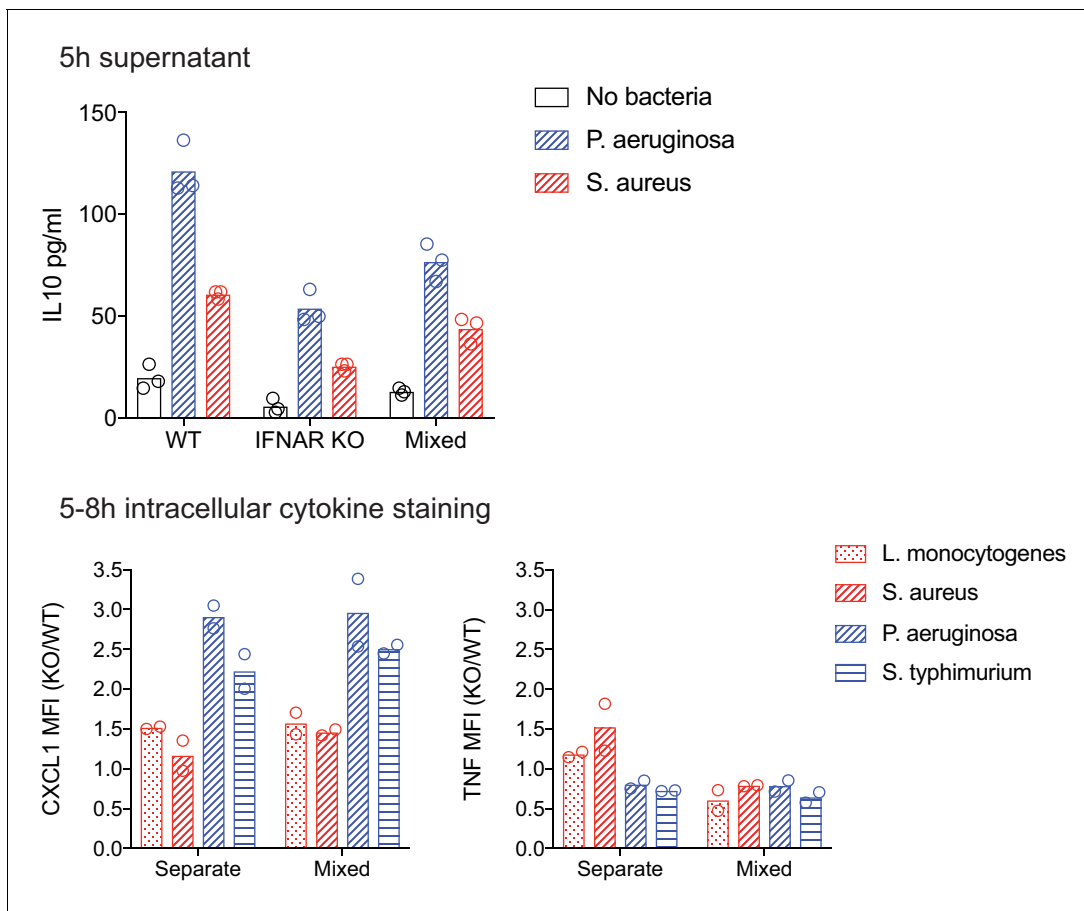


**Figure 6—figure supplement 3.** The impact of IL10 deficiency or addition on TNF and CXCL1 shutdown. WT, *Ifnar1*<sup>-/-</sup>, and *Il10rb*<sup>-/-</sup> BMDM were stimulated with heat-killed *P. aeruginosa* or *S. aureus*, as indicated. After 6 hr of stimulation, supernatants were removed (0–6 hr) and replaced with fresh medium for a subsequent 6 hr of stimulation (6–12 hr). TNF and CXCL1 were quantified using cytometric bead array. Blocking antibody to IL-10R (10 µg/mL; 30 min pre-stimulation) or recombinant IL-10 (1 ng/mL, 2 hr post-stimulation) was added to select conditions, where indicated. Arrows are to Figure 6—figure supplement 3 continued on next page

*Figure 6—figure supplement 3 continued*

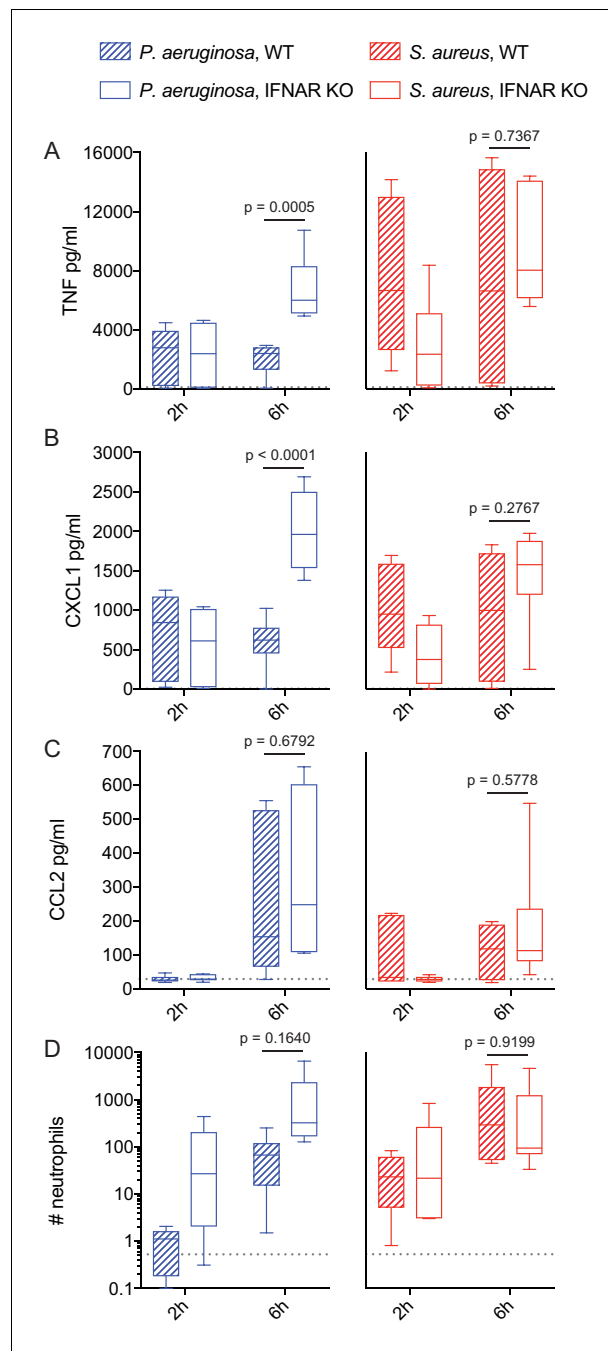
guide the eye to shutdown from the early to late timepoint. The mean values for unstimulated WT BMDM (0–6 hr) are shown as dotted lines. In some cases, these controls are not visible because they are below the level of detection (a zero value). Data points represent replicate wells and results are representative of two independent experiments.

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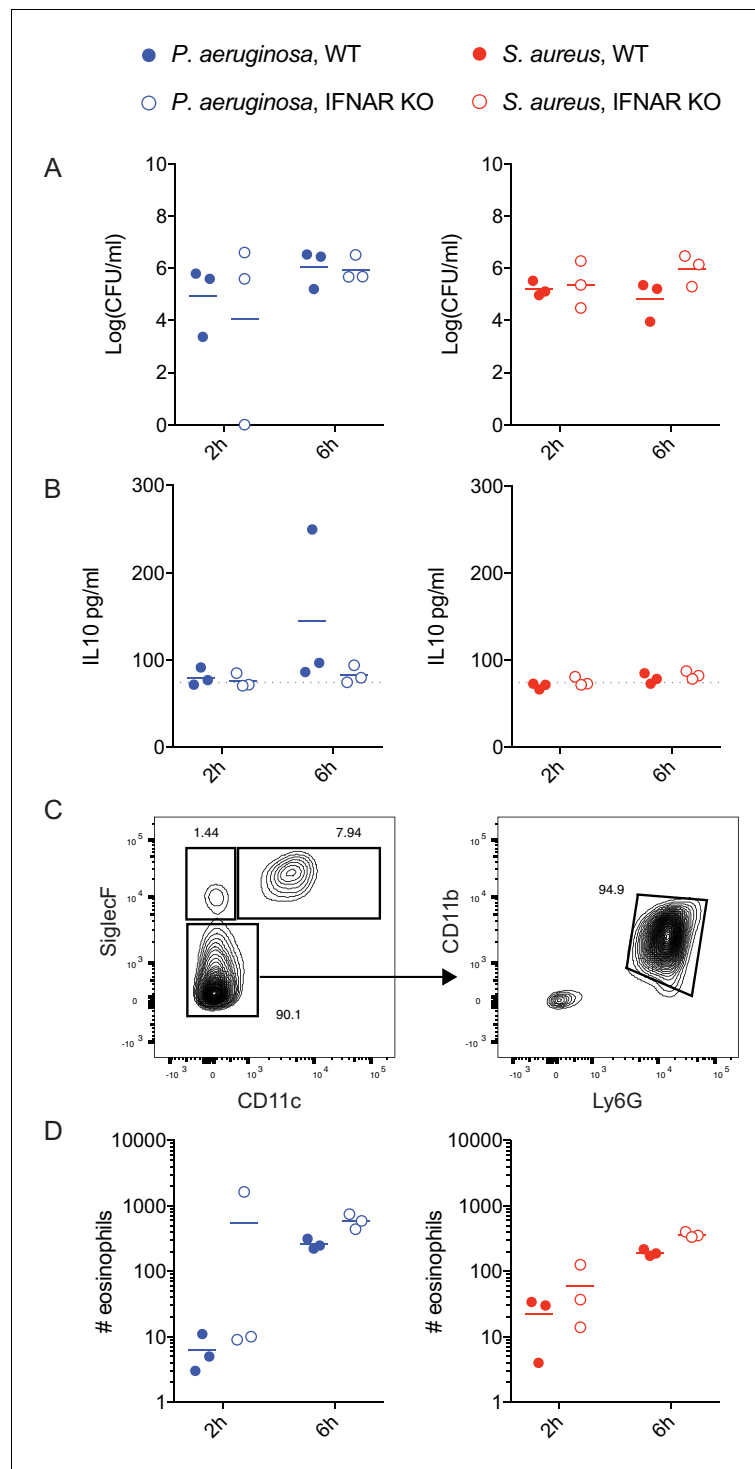
**Figure 6—figure supplement 4.** Characterization of mixed WT and *Ifnar1*<sup>-/-</sup> BMDM cultures. As in **Figure 6F and G**, WT and IFNAR KO BMDM were stimulated with the indicated heat-killed bacteria, separately or in mixed BMDM cultures (1:1 of WT:KO). After 5 hr of stimulation, either supernatants were collected for IL-10 quantification by cytometric bead array (above) or BFA was added for the final 3 hr of an 8 hr stimulation (below). CXCL1 and TNF were quantified by intracellular cytokine staining and flow cytometry and the MFI is shown as a KO/WT ratio. Data points represent replicate wells and results are representative of two or more independent experiments. .

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**Figure 7.** IFN-mediated negative feedback shapes in vivo inflammatory responses in a pathogen-specific manner. WT or *Ifnar1*<sup>-/-</sup> littermate mice were infected intranasally with  $5 \times 10^7$  CFU of *P. aeruginosa* or *S. aureus*, as indicated. At 2 and 6 hr after infection, mice were sacrificed and BAL was collected for quantification of cytokines by CBA (A–C) and neutrophils by flow cytometry (D). Data are pooled from two independent experiments, with a total of 6 mice per experimental group. Neutrophil numbers were normalized to the mean of WT *P. aeruginosa* infected mice for each independent experiment. The mean values for PBS treated control mice are shown as dotted lines.

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**Figure 7—figure supplement 1.** Lung infection with *P. aeruginosa* and *S. aureus*. WT or *Ifnar1*<sup>-/-</sup> littermate mice were infected intranasally with  $5 \times 10^7$  CFU of *P. aeruginosa* or *S. aureus*, and mice were sacrificed at 2- and 6 hr post-infection. CFU were determined by plating dilutions of homogenized whole lungs. IL-10 was quantified from BAL using CBA. Representative flow cytometry gating shows CD11b+Ly6G+ neutrophils shown in **Figure 7D** and SiglecF+CD11c- eosinophils shown below. Data are shown for one of two experiments; IL-10 was not detected above baseline in the other experiment.

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