Co-stimulation with opposing macrophage polarization cues leads to orthogonal secretion programs in individual cells

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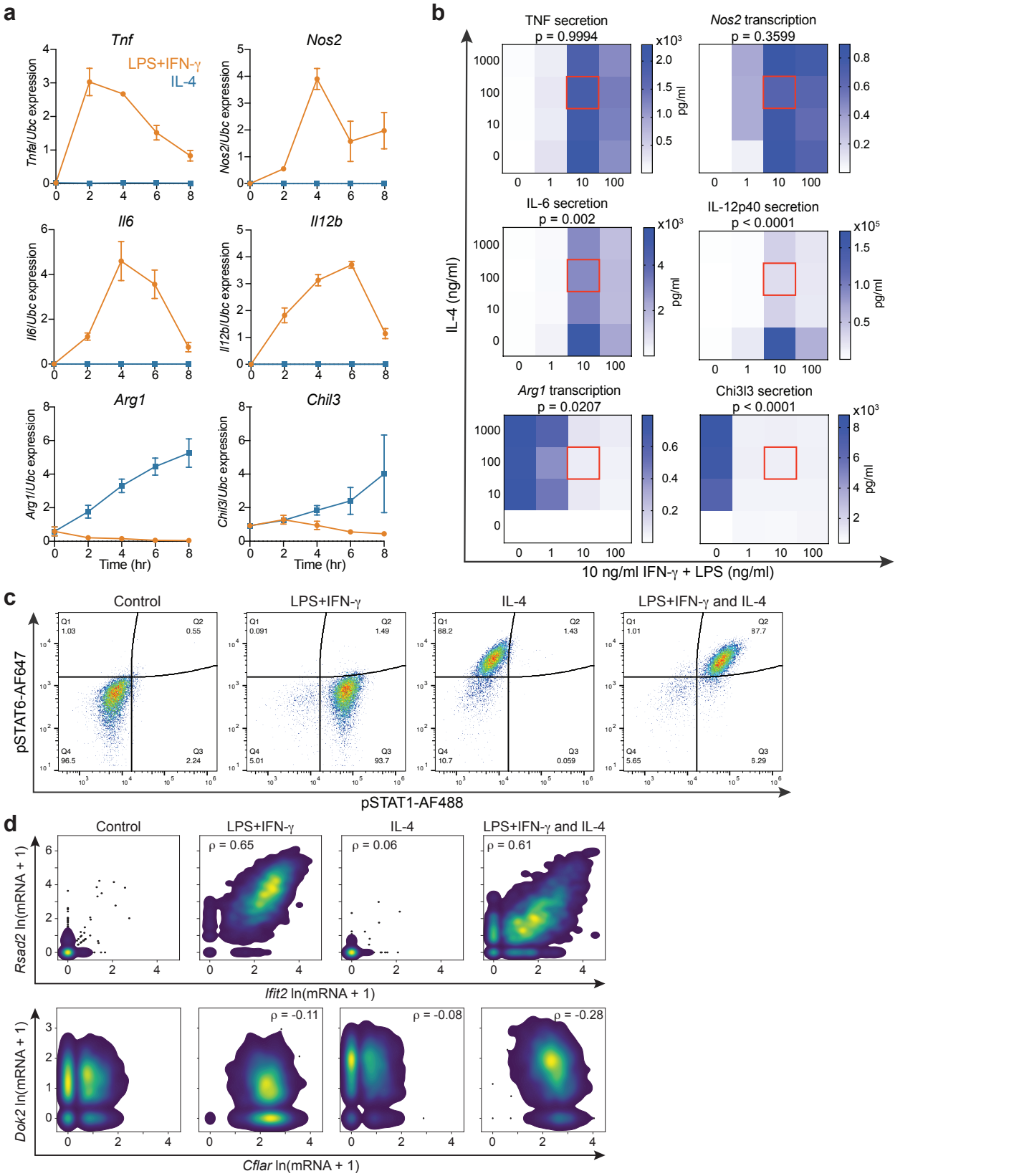
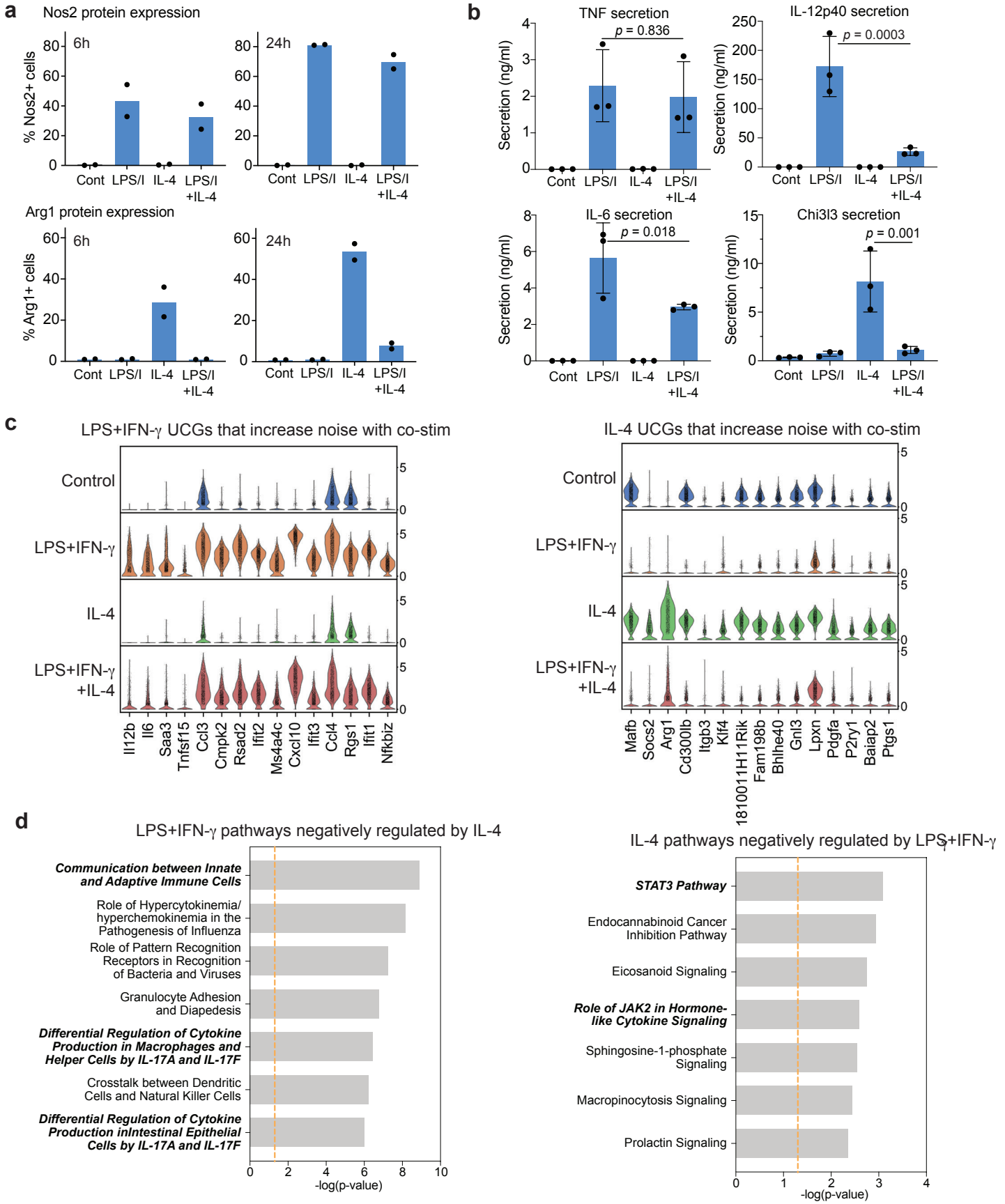
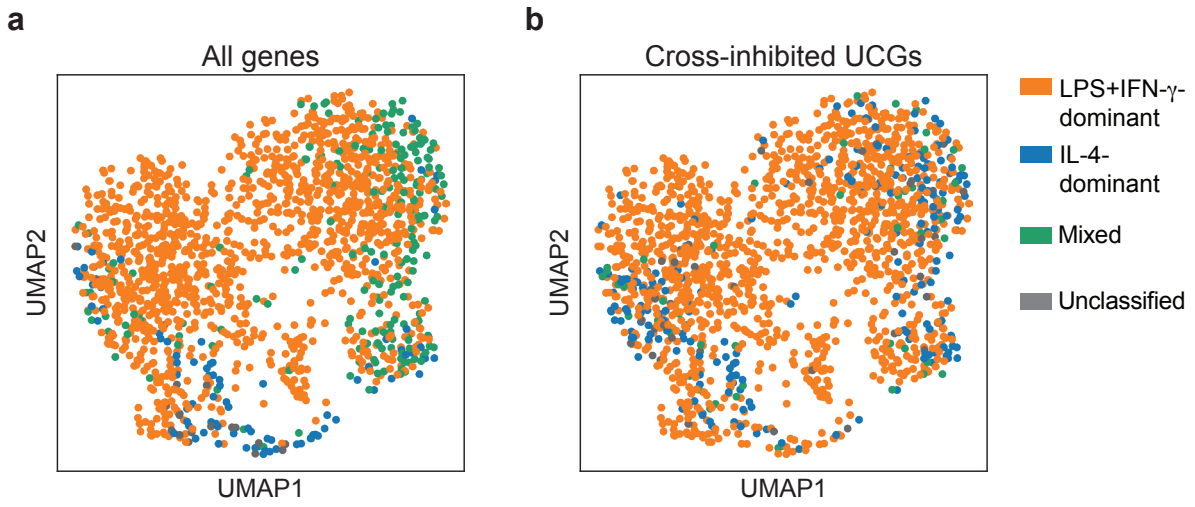
Supplementary Information



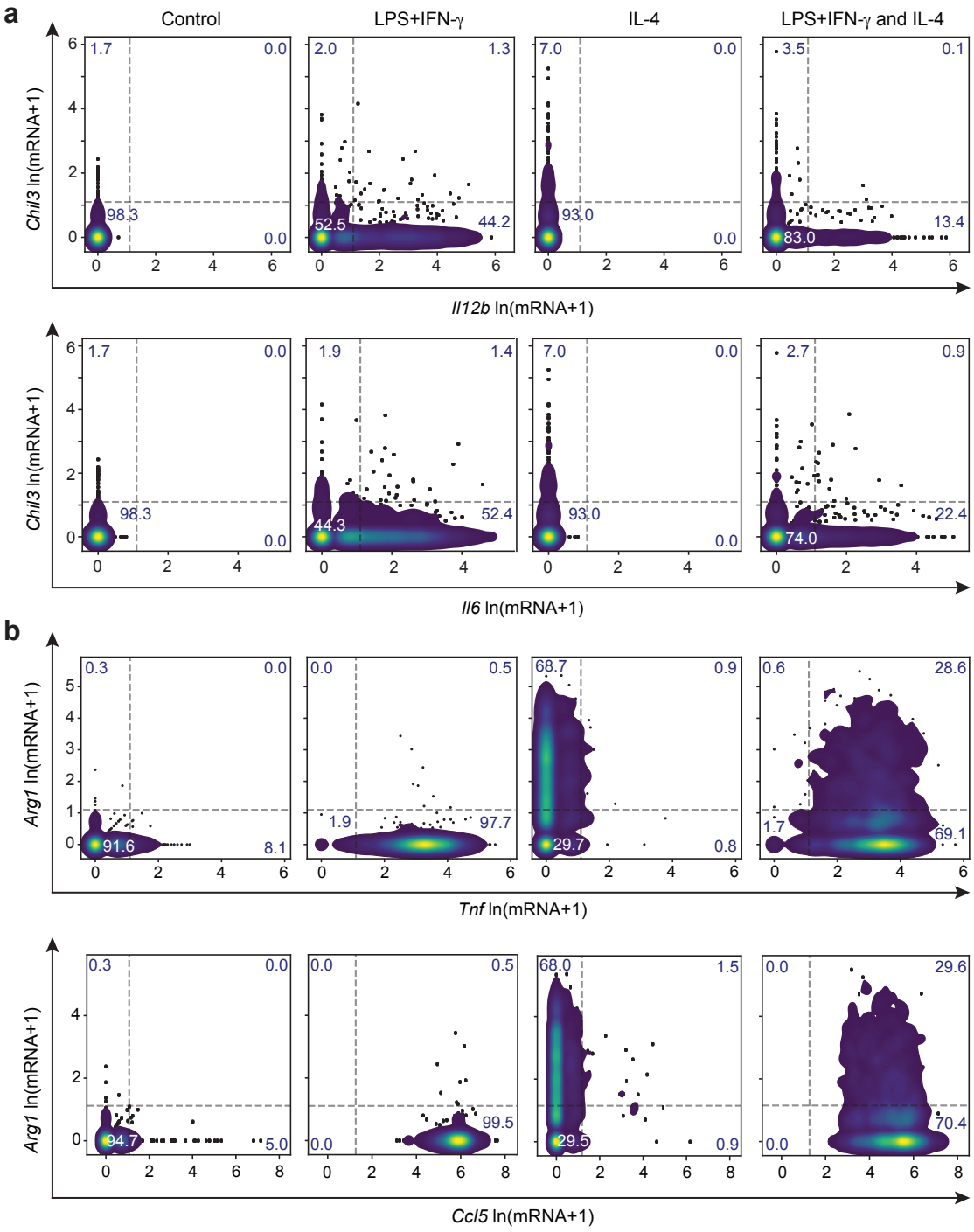
Supplementary Fig. 1 (associated with Fig. 1). Timing and dose dependence of cross-inhibition between LPS+IFN-γ and IL-4 responses in BMDMs. a Time course of transcription in BMDMs after stimulation with 10 ng/ml LPS+10 ng/ml IFN-γ or 100 ng/ml IL-4 for selected targets measured by RT-qPCR (mean ± SEM, n=3 biological replicates). **b** Heatmaps of response in BMDMs after stimulation for 24 h with 10 ng/ml IFN-γ + a range of LPS doses combined with a range of doses of IL-4. Data represent *Arg1* and *Nos2* transcript levels measured by RT-qPCR and Chi3l3, IL-6, IL-12p40, and TNF protein levels measured by ELISA (mean, n=3 biological replicates). *P*-value represents the significance of the interaction between the stimulations calculated by a 2-way ANOVA. **c** Phospho-Stat1 and phospho-Stat6 expression by flow cytometry in BMDMs after stimulation with 10 ng/ml LPS+10 ng/ml IFN-γ, 100 ng/ml IL-4, or the combination for 30 min. **d** Density scatter plots of scRNA-seq transcript counts across individual cells for *Rsad2* vs. *Ifit2* (top) and *Dok2* vs. *Cflar* (bottom). ρ indicates Spearman correlation coefficient. Gene expression is shown as ln(transcript count +1). Source data are provided as a Source Data file.



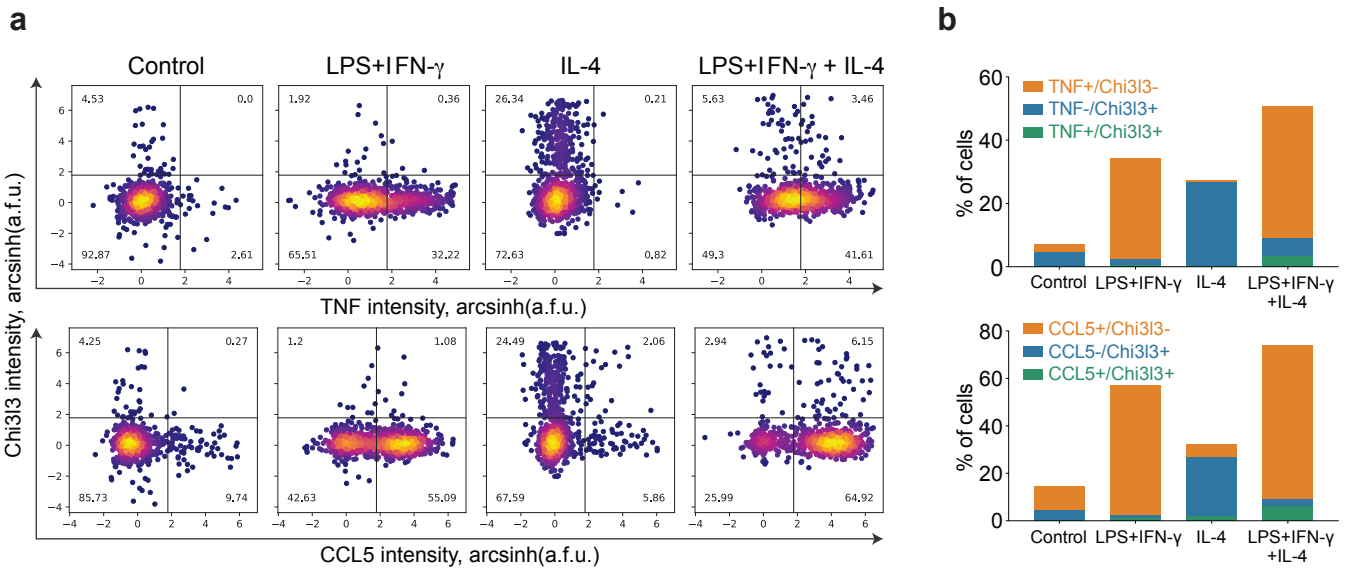
Supplementary Fig. 2 (associated with Fig. 2). Cross-inhibition and cell-to-cell noise in gene expression after co-stimulation. **a** Levels of Nos2 (top) and Arg1 (bottom) protein detected by flow cytometry after stimulation for 6 h (left) and 24 h (right) with 10 ng/ml LPS+10 ng/ml IFN-γ, 100 ng/ml IL-4, or the combination (mean, n=2 biological replicates). **b** Levels of TNF, IL-12p40, IL-6 and Chi3l3 secretion after stimulation for 24 h with 10 ng/ml LPS+10 ng/ml IFN-γ, 100 ng/ml IL-4, or the combination (mean ± SEM, n=3 biological replicates). P-values determined with two-sided one-way ANOVA with Sidak correction for multiple comparisons. **c** Violin plots of scRNA-seq measurements of LPS+IFN-γ-induced (top) and IL-4-induced (bottom) genes with the highest change in cell-to-cell gene expression noise measured by Fano factor. **d** Result from Ingenuity Pathway Analysis listing the top 7 canonical pathways that are associated with the set of LPS+IFN-γ-induced (top) or IL-4-induced (bottom) genes that are inhibited after co-stimulation. *P*-values indicate the significance of the overlap between the canonical pathway and the inhibited genes (determined by one-sided Fisher's exact test). Source data are provided as a Source Data file.



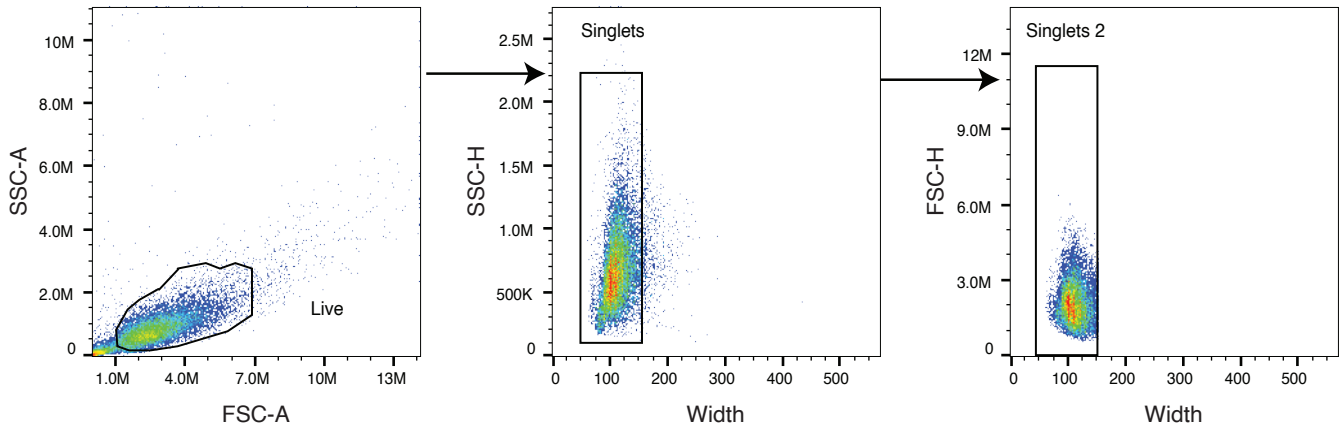
Supplementary Fig. 3 (associated with Fig 3). Neural network classifier results identify clusters of mixed and single-stimuli-dominant cells. a-b UMAP representation of classification results from the neural network classifier trained on all genes (**a**) or UCGs that displayed cross-inhibition after co-stimulation (**b**).



Supplementary Fig. 4 (associated with Fig. 4). Orthogonal expression is observed for *Il6* and *Il12b* with *Chil3* but not for *Tnf* and *Ccl5* with *Arg1*. Density scatter plots of scRNA-seq transcript counts across individual cells for *Chil3* vs. *Il12b* (**a**, top) and *Il6* (**a**, bottom); and for *Arg1* vs. *Tnf* (**b**, top) and *Ccl5* (**b**, bottom). Data is represented as ln(transcript count +1).



Supplementary Fig. 5 (associated with Fig. 5). TNF and CCL5 are not orthogonally secreted with Chi3l3. A multiplexed single-cell secretion assay was used to measure cytokine/chemokine production in individual BMDMs stimulated for 48 h with media alone, 10 ng/ml LPS+10ng/ml IFN-γ, 100 ng/ml IL-4, or both. **a** Density scatter plots of single-cell secretion intensity (a.f.u.) across individual cells for Chi3l3 vs TNF (top) and Chi3l3 vs CCL5 (bottom). **b** Quantification of single-positive and double-positive cells after 48-hour co-stimulation for secretion of Chi3l3 and TNF (top) and Chi3l3 and CCL5 (bottom). Data is pooled from 2 biological replicates.



Supplementary Fig. 6. Gating strategy for flow cytometry experiments. Gating strategy for gating of live, singlet BMDMs.

Supplementary Table 1. Capture and detection ELISA antibody pairs for secretion profiling.

Antibody Pair	Vendor	Catalog No.
TNF	eBioscience	88-7324-88
CCL17	R&D	DY529
IL-12p40	BD Biosciences	555165
IL-10	BD Biosciences	555252
MMP9	R&D	DY6718
IL-6	R&D	DY406
IGF-I	R&D	DY791
CCL2	R&D	DY479
CCL8	R&D	DY790
Chi3l3	R&D	DY2446
CCL3	R&D	DY450
CXCL1	R&D	DY453
GM-CSF	R&D	DY415
CCL22	R&D	DY439
CCL5	R&D	DY478

Supplementary Table 2. Primers used for qPCR.

Target	Forward Primer (5’ to 3’)	Reverse Primer (5’ to 3’)
Nos2	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
Klf4	GACTAACCGTTGGCGTGAGG	GTCTAGGTCCAGGAGGTCGT
Nfkbiz	GAGTCCCGTCCCAGAGGTG	ACTCTGTGTCTTAAACTCATCCAC
Il6	TCCTCTCTGCAAGAGACTTCC	TTGTGAAGTAGGGAAGGCCG
Tnf	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
Il12b	CGCCACACAAATGGATGCAA	TGTGTCCTGAGGTAGCCGTA
Chil3	CAGGTCTGGCAATTCTTCTGAA	GTCTTGCTCATGTGTGTAAGTGA
Ubc	ACCACCAAGAAGGTCAAACAGG	TAAGACACCTCCCCCATCAC