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Supplementary Materials for

Analysis of single-cell cytokine secretion reveals a role for paracrine signaling in coordinating macrophage responses to TLR4 stimulation

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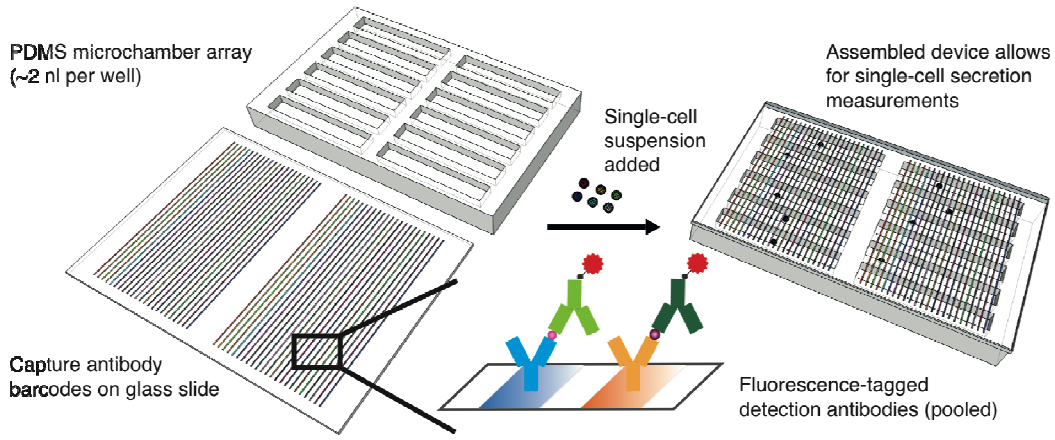

Fig. S1. Schematic of the SCBC. A PDMS array of 3,080 wells is loaded with cells by pipet. Cells are allowed to settle into the wells by gravity resulting in an approximately Poisson distribution of 0, 1, 2, or 3+ cells per well. Cells are then covered with a glass slide that was previously patterned with capture antibodies for the targets of interest (8 in this study). Cells are incubated in the device for up to 20 hours and then the antibody-patterned glass slide is removed and incubated with fluorescently labeled detection antibodies to measure the secretion intensity per cell. The zero-cell wells are used to set the background threshold (BT) of secretion for each cytokine. Only wells containing single cells were included in this study. Figure is adapted from (*13*).

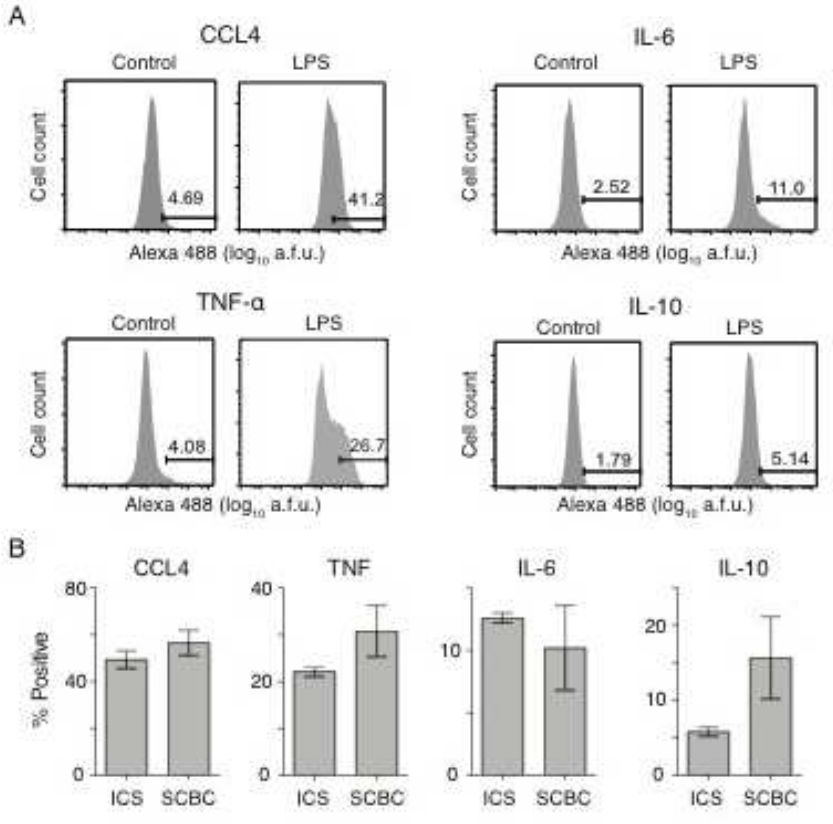

Fig. S2. Intracellular cytokine staining is consistent with the results from the SCBC assay. (**A** and **B**) Brefeldin A was added to U937 cells in the presence or absence of LPS (100 ng/ml). Cells were incubated for 20 hours, fixed, immunostained, and analyzed by flow cytometry. (A) Representative flow cytometry histograms after intracellular cytokine staining (ICS). The bars indicate the gates and the percentages of cells positive for the indicated cytokines. (B) Comparison of the percentages of LPS-stimulated cells that were positive for the indicated cytokines as measured by ICS versus in the SCBC. Data are means ± SEM of two (ICS) or four (SCBC) biological replicates. Means are not statistically significantly different ($P > 0.05$ by Student's *t* test).

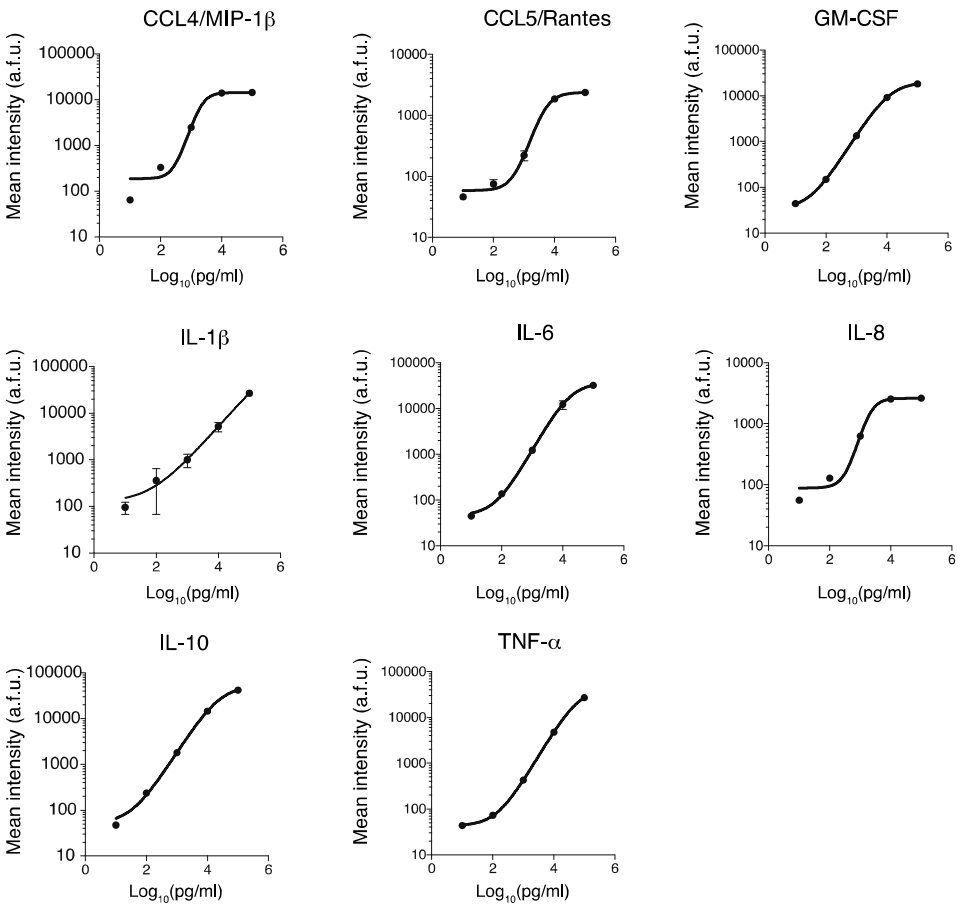

Fig. S3. Recombinant protein standard curves provide a means to convert intensities measured in the SCBC assay to protein concentrations. Recombinant proteins were incubated with flow-patterned antibodies at a range of concentrations, and then were analyzed for intensity according to the same method as used for the SCBC. The resulting standard curve was fit to a four-parameter logistic curve. The recombinant standard curves from two independent experiments were combined and a common standard curve was then used to convert single-cell intensities in the SCBC to single-cell protein concentrations (see the Materials and Methods for details).

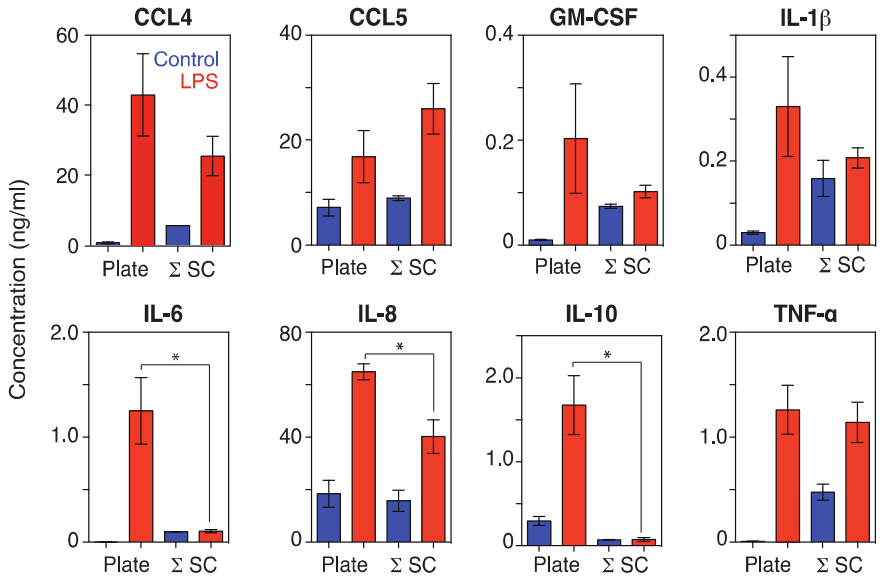

Fig. S4. Including cells below the BT does not change the statistically significant differences between total secretion in the cultured cell population and the SCBC population. Average secretion of the indicated cytokines in the cell population (plate) and of all single cells cultured in the SCBC (Σ SC) for vehicle-treated (Control, blue) and LPS-treated (red) cells. Cell-population concentrations of secreted cytokines are the same as those in Fig. 1C. Single-cell secretion intensities were converted based on recombinant protein standard curves (see fig. S3 and the Materials and Methods), and include all cells with detectable secretion based on the lower limit of the standard curve (that is, all cells above the detection threshold (DT) were included even if the cells were below the BT). Values are means ± SEM of two (control) or four (LPS) biological replicates. *$P < 0.05$ by Student's t test.

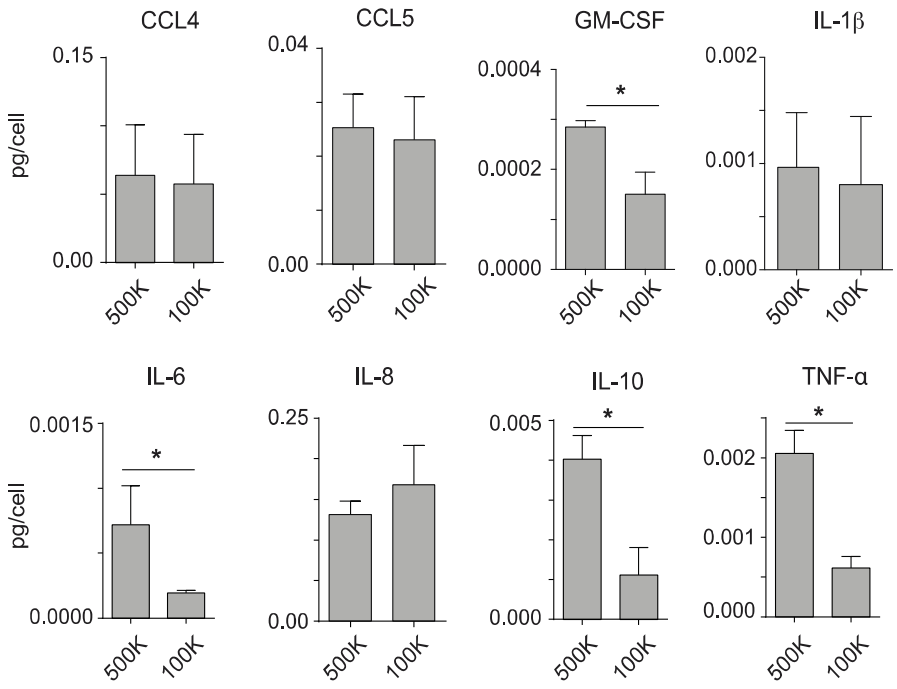

Fig. S5. Changing cell density affects the amount of cytokine secreted per cell for a subset of cytokines, including IL-6 and IL-10. The amounts of the indicated cytokines secreted by LPS-treated U937 cells that were incubated at two different cell densities (5 or 1×10^5 cells/ml) were measured by ELISA. The concentration of cytokine per cell (pg/cell) was calculated by dividing the total protein concentration (pg/ml) by the cell density (cell/ml). Data are means ± SD of three biological replicates. **P* < 0.05 by Student's *t* test.

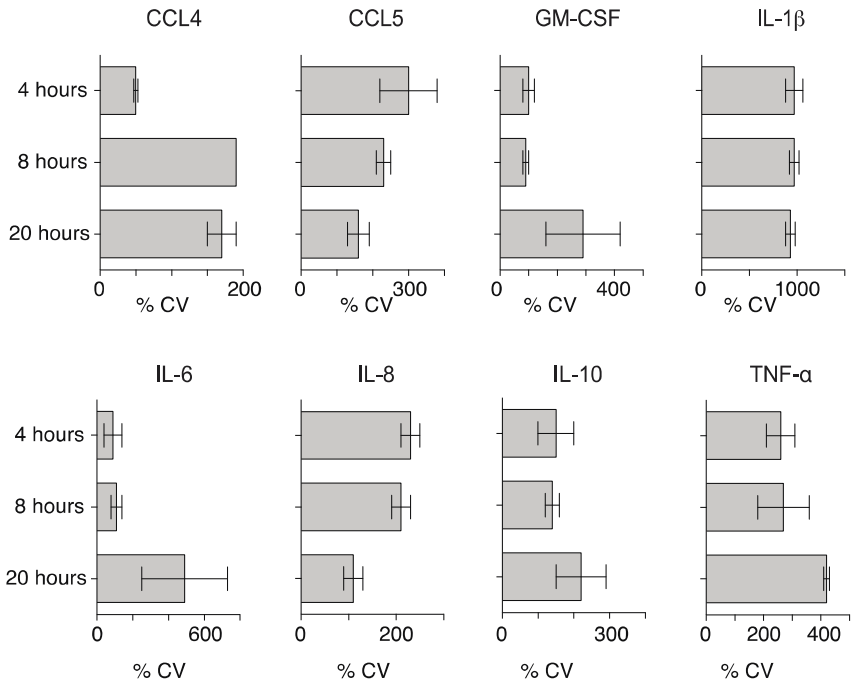

Fig. S6. The secretion noise of IL-6 and GM-CSF increases with time in the SCBC. The CV for each of the indicated cytokines was calculated for each SCBC experiment by taking the ratio of the SD to the mean of the concentration secreted by all single cells. Data are means ± SEM of two independent SCBC experiments for each time point.

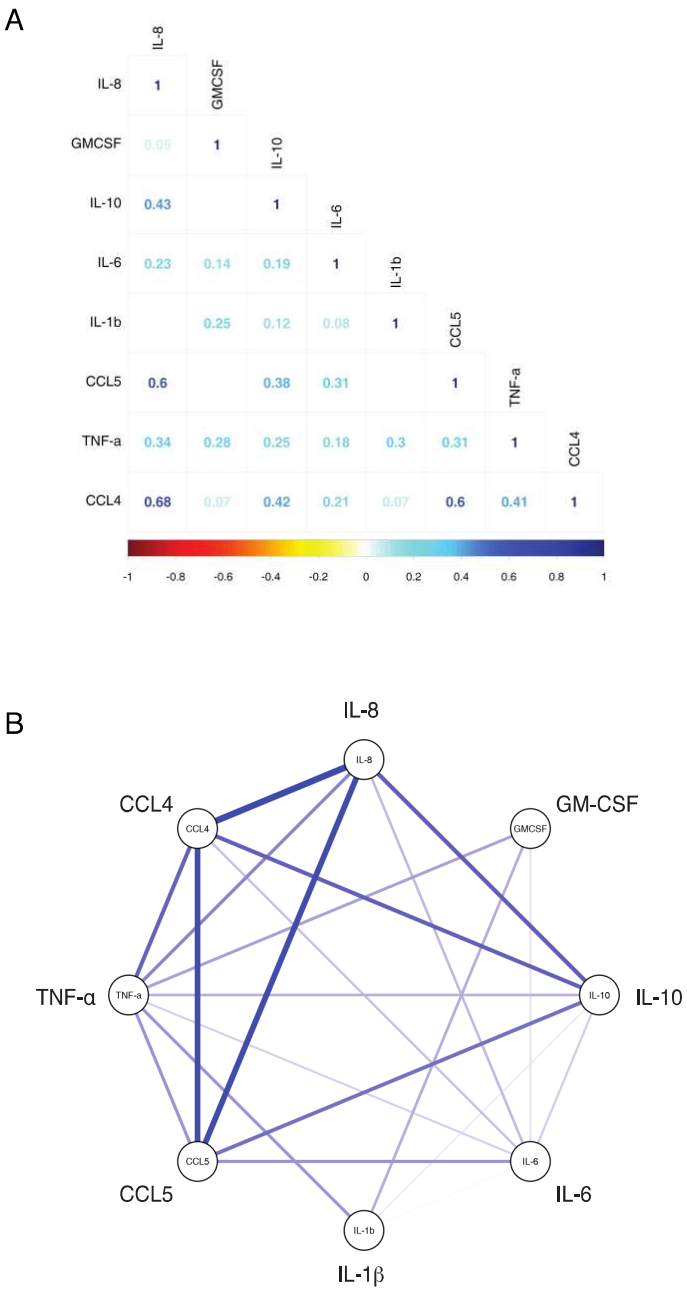

Fig. S7. Pairwise correlations do not reliably identify candidates for paracrine signals. (**A**) Statistically significant pairwise correlations ($P < 0.05$) for all secreted protein pairs computed from the single-cell time course data that was summarized in Fig. 2, A and B. (**B**) Network diagram of all the statistically significant pairwise correlations calculated in (A).

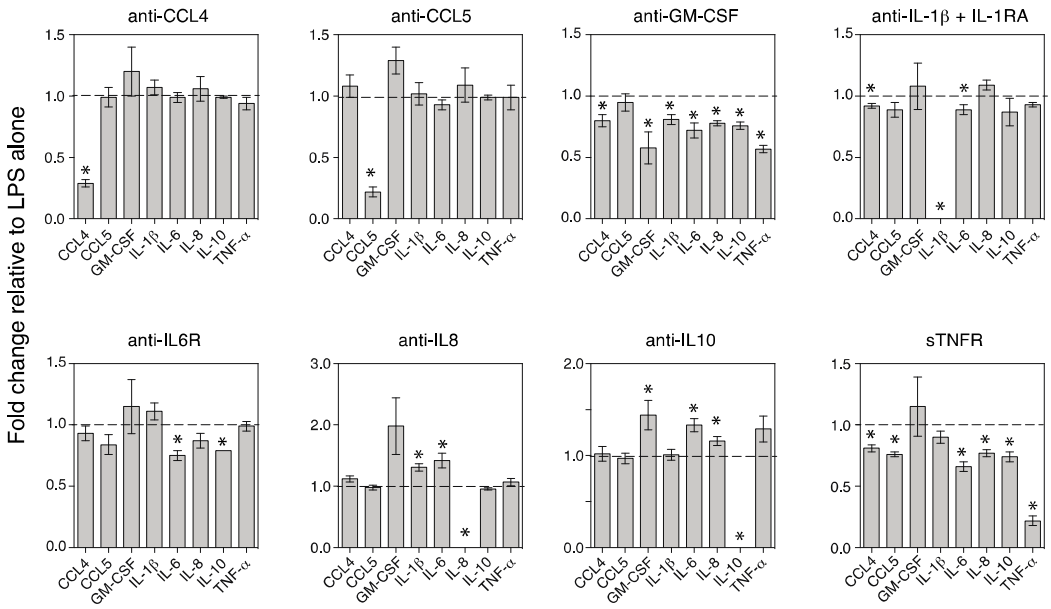

Fig. S8. Analysis of the changes in the LPS-stimulated secretion at 20 hours of the eight cytokines that were caused by blocking each signal individually in a cultured cell population. The amounts of the indicated proteins that were secreted were measured by ELISA or Bio-plex. Data are means ± SEM of three biological replicates. **P* < 0.05 by *t* test. These data are summarized in a blocking matrix in Fig. 3C. Note that in all cases, the neutralization antibody or soluble receptor (for TNF) statistically significantly reduced detection of the respective cytokine, except in the case of IL-6. For IL-6, the blocking antibody targets IL-6R, and therefore the observed reduction in IL-6 abundance is a functional response to a loss of signaling through the IL-6R. See table S2 for list of blocking antibodies and targets.

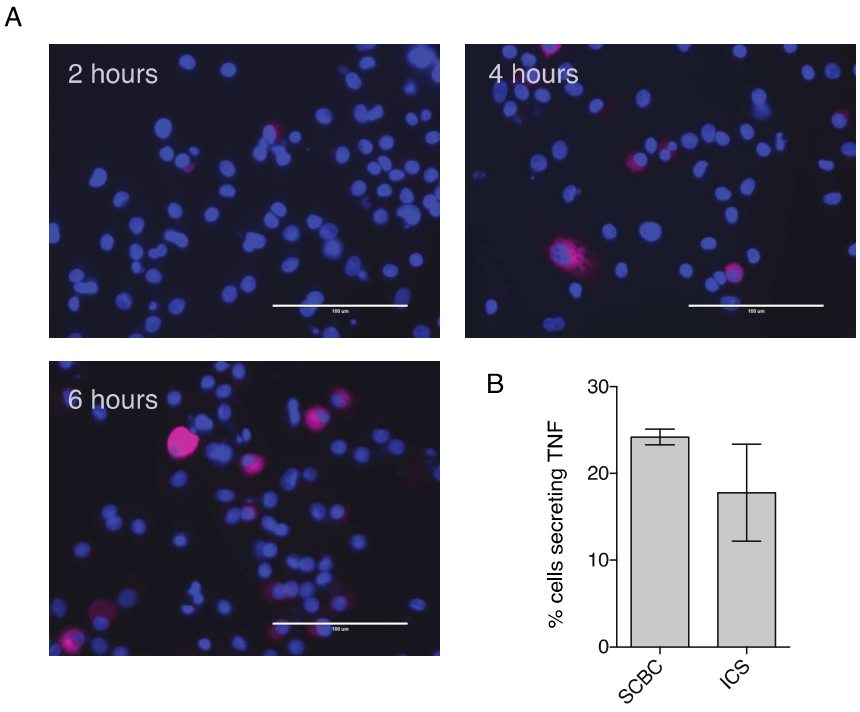

Fig. S9. Intracellular staining for TNF-α demonstrates that high secretors exist in a cell population. (**A**) Intracellular staining for TNF-α at 2, 4, and 6 hours after LPS stimulation in the presence of brefeldin A. (**B**) Comparison of the percentages of cells positive for TNF-α at 4 hours after LPS stimulation as quantified in the SCBC and by ICS. Data are means ± SEM of two biological replicates and were not statistically significantly different ($P > 0.05$ by Student's *t* test).

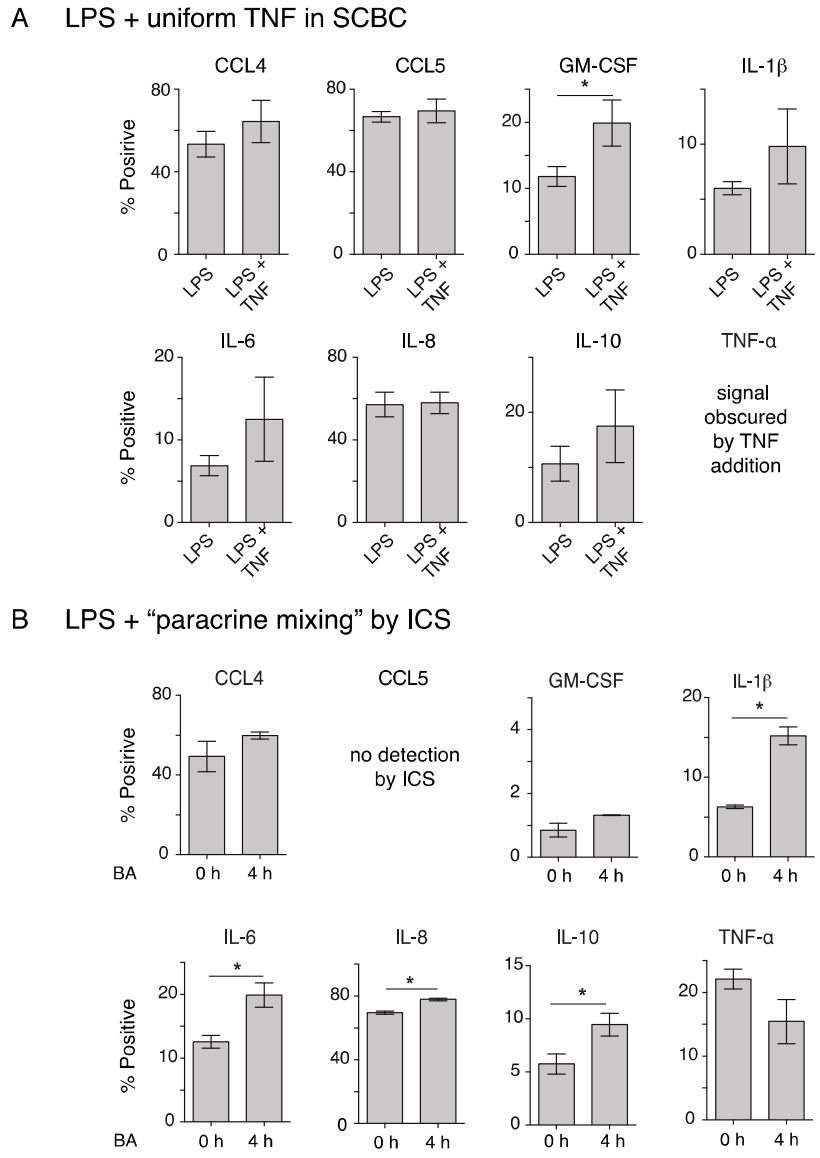

Fig. S10. TNF-α and paracrine mixing increase the percentage of single cells positive for IL-6 and IL-10 in response to LPS in the absence of paracrine signaling. (**A**) U937 cells were stimulated with LPS (100 ng/ml) in the presence or absence of TNF-α (1 ng/ml) before being loaded into the SCBC. Cells were incubated for 20 hours, and the percentage of cells secreting the indicated cytokines at concentrations above the DT was determined. Data are means ± SEM of two biological replicates. Note that the y-axes have different scales. **P* < 0.05 by one-sided, paired *t* test. (**B**) U937 cells were stimulated with LPS, and brefeldin A was added to the culture medium at 0 or 4 hours. Cells were collected at 20 hours, labeled for the protein of interest, and analyzed by flow cytometry. Data are means ± SD of two biological replicates. **P* < 0.05 by *t* test.

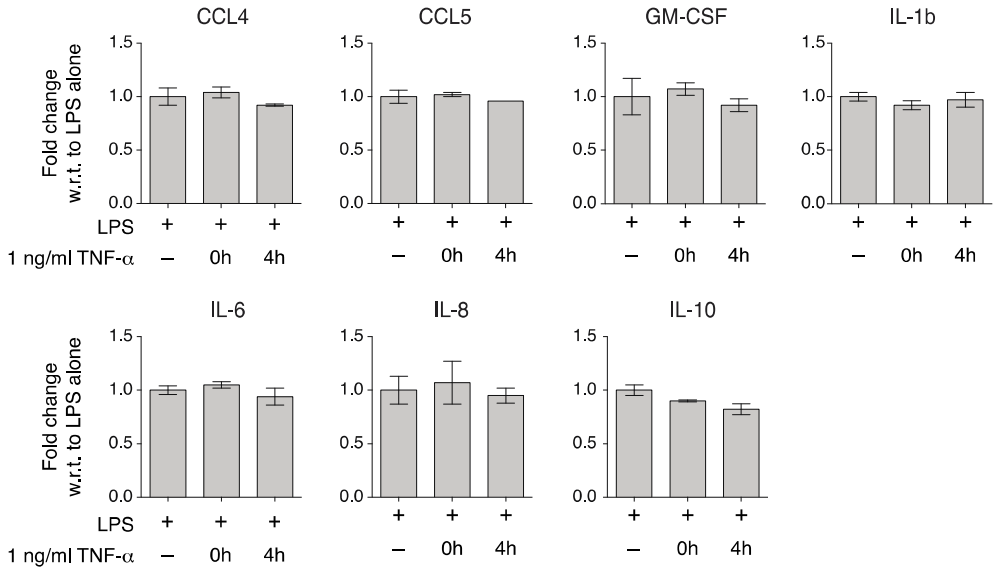

Fig. S11. The timing of TNF-α addition does not enhance LPS-induced cytokine secretion in a cultured cell population. U937 cells were stimulated with LPS (100 ng/ml) alone or in combination with TNF-α (1 ng/ml), which was added either at the same time as the LPS or 4 hours later. The cells were then cultured for 20 hours. The amounts of indicated proteins that were secreted were measured by Bio-plex, and are expressed as the mean of the fold-change (LPS+TNF relative to LPS alone) ± SEM in cytokine secretion of two biological replicates. The means are not statistically significantly different ($P > 0.05$ by Student's *t* test).

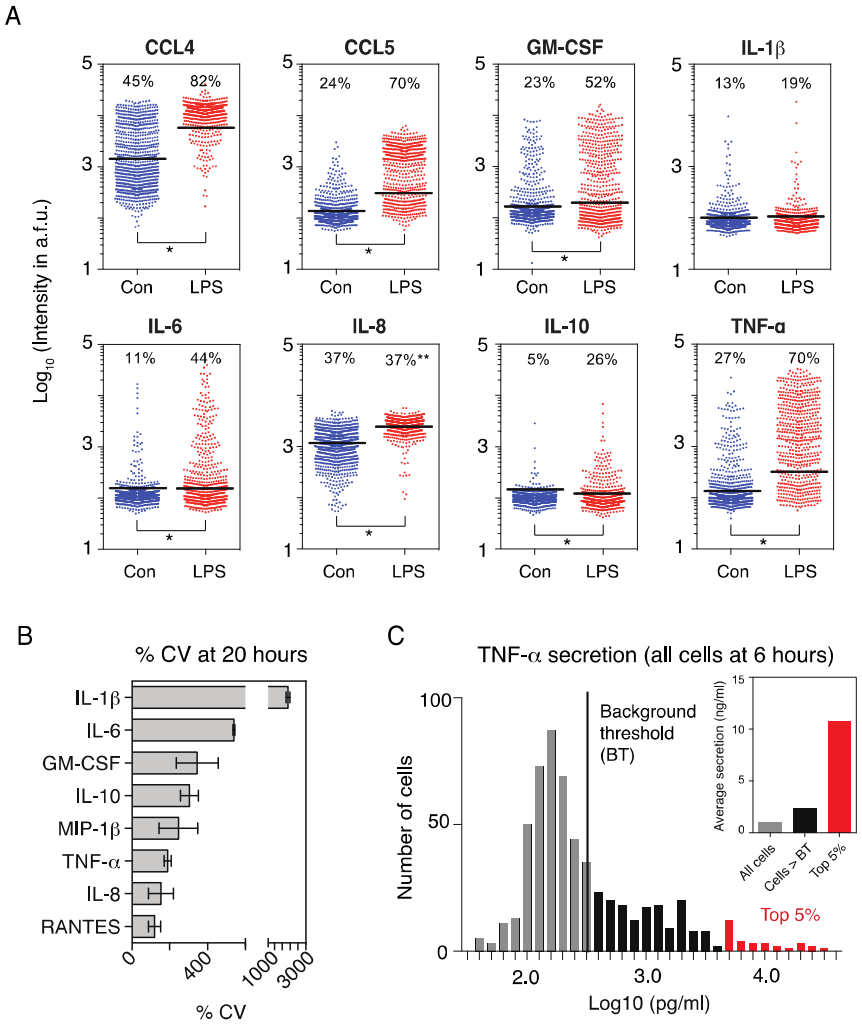

Fig. S12. The cell-to-cell heterogeneity in cytokine secretion of primary MDMs is similar to that of U937 cells. (**A**) Secreted protein intensities (a.f.u.) for vehicle-treated (blue; n = 906 cells) and LPS-stimulated (red; n = 612 cells) single MDMs for 20 hours after stimulation. The mean secretion intensity for all cells is indicated by the black line, and the percentage of cells that secreted the indicated proteins are shown. **P* < 0.05 by Bonferroni-corrected Wilcoxon–Mann–Whitney test. **The detection threshold for IL-8 in the LPS-stimulated condition is twice that of the control because of the high background for IL-8, and therefore the percentage of cells that secreted IL-8 is less than the actual number. Data are from one of two independent experiments. (**B**) Percentage coefficient of variation (% CV) of protein secretion for all cells 20 hours after stimulation with LPS. Data are averages ± SEM of two biological replicates. (**C**) Distribution of single-cell secretion of TNF-α 6 hours after stimulation with LPS (n = 580 cells). The BT is 283 ng/ml (black line). Cells above the BT are in black, and the top 5% of secreting cells are in red. (Inset) Average secretion for all cells (gray), cells above the DT (black), and top 5% of cells (red). The top 5% of secreting cells account for 54% of all the TNF-α protein that was secreted.

Table S1. Scientific support for the potential autocrine or paracrine activation of and by the secretion of the panel of cytokines analyzed in this study. The table lists which cytokines are potentially regulated (middle) by another cytokine acting as the ligand (left).

Ligand	Regulated cytokines	References
IL-1β	IL-8, TNF-α, IL-6, IL-10	(*22*, *50-52*)
GM-CSF	IL-8, IL-1β, TNF-α	(*28*, *53*)
IL-6	IL-1RA, IL-10	(*54*) (*55*)
MIP-1β/CCL4	IL-1β, TNF-α	(*56*)
RANTES/CCL5	TNF-α, IL-6, IL-10	(*57*)
IL-10	IL-1α, IL-1β, IL-6, IL-8, TNF-α, GM-CSF, and G-CSF, IFN-β	(*21*, *32*, *33*)
TNF-α	IL-8, IL-6, GM-CSF, IL-10	(*52*, *58-60*)
IL-8	IL-1β	(*61*)

Table S2. Neutralizing antibodies that were used to block signaling in the cell population studies.

Blocking agents	Manufacturer (Catalog No.)	Concentration	Target
Recombinant TNF receptor (rTNFR)	R&D (636-R1-025)	5 μg/ml	TNF-α
Recombinant IL-1RA	Genscript (Z00367-10)	0.5 μg/ml	IL-1R
Anti-IL1β	R&D (MAB201)	1 μg/ml	IL-1β
Anti-IL-6 receptor α	R&D (AB-227-NA)	1 μg/ml	IL-6 receptor α
Anti-GM-CSF	R&D MAB215	5 μg/ml	GM-CSF
Anti-IL-10	BD (554495)	10 μg/ml	IL-10
Anti-IL-8	R&D (MAB208)	5 μg/ml	IL-8
Anti-CCL4	R&D (MAB 271)	5 μg/ml	CCL-4
Anti-CCL5	R&D (MAB678)	5 μg/ml	CCL-5

Table S3. ELISA antibody pairs used in the SCBC.

Target	Capture antibody	Detection antibody
TNF-α	eBioscience, 14-7348	eBioscience, 13-7349
IL-1β	eBioscience, 16-7018	eBioscience, 13-7016
RANTES/CCL5	R&D, DY278	R&D, DY278
IL-6	eBioscience, 14-7068	eBioscience, 13-7068
IL-10	eBioscience, 14-7108	eBioscience, 13-7109
GMCSF	eBioscience, 16-7336	Biolegend, 502304
IL-8	R&D, DY208	R&D, DY208
MIP-1β/CCL4	R&D, DY271	R&D, DY271