ISCI, Volume *20*

Supplemental Information

A Nanoparticle-Based Affinity Sensor that Identifies and Selects Highly Cytokine-Secreting Cells

Guozhen Liu, Christina Bursill, Siân P. Cartland, Ayad G. Anwer, Lindsay M. Parker, Kaixin Zhang, Shilun Feng, Meng He, David W. Inglis, Mary M. Kavurma, Mark R. Hutchinson, and Ewa M. Goldys

Supplementary Information

A nanoparticle-based affinity sensor that identifies and selects highly cytokine-secreting cells

Guozhen Liu, Christina Bursill, Siân P. Cartland, Ayad G. Anwer, Lindsay M. Parker, Kaixin Zhang, Shilun Feng, Meng He, David W. Inglis, Mary M. Kavurma, Mark R. Hutchinson, Ewa M. Goldys

Transparent Methods

Preparation of superparamagnetic fluorescent beads labeled with IL-6 detection antibody

Superparamagnetic beads (SPIO) were selected for conjugation with the IL-6 antibody (CCC). Carboxylated superparamagnetic iron oxide particles (SPIO, 1% solid, 10 mg/mL, ~0.9 µm, Bangs Laboratories, USA) incorporating Dragon Green fluorophore (ex480, em520), (1 mg) were dispersed in 1 mL of 100 mM MES buffer at pH 5.2. This dispersion was then mixed with 3.2 mg EDC (1-ethyl-3-[3 dimethylaminopropyl]carbodiimide hydrochloride, Thermo Scientific) and 1.8 mg NHS (N-hydroxysuccinimide) and vortexed at room temperature for 30 min. After which the pH was adjusted to 8.0. The 20 µL anti-IL-6 monoclonal antibody catalog No. MAB406 from R&D Systems (0.5 mg mL^{-1} in 1xPBS) was added to the solution immediately, and stirred on a nonmagnetic mixing device for 2 h at room temperature. The resulting antibody-beads conjugates were magnetically separated by placing a magnet under the bottom of the reaction vessel, and the supernatant was discarded. Finally, the antibody modified SPIO (SPIO_Ab) was separated and washed with washing buffer (0.1 M 10xPBS, pH7.4) three times. The obtained SPIO_Ab was redispersed in 0.5 mL PBS and stored at 4 ^{o}C. The ratio equates to 10 µg antibody per mg beads. The same protocol was used for making superparamagnetic fluorescent beads labeled with IL-1β detection antibody except replacing anti-IL-6 monoclonal antibody by anti-IL-1β monoclonal antibody.

Sample preparation for confocal imaging and fluorescence staining

Cells were imaged using a SP2 (Leica Microsystems) confocal microscope. Cells were harvested after two washes with PBS, then pelleted at 1200 g for 5 min. Cells were then suspended in PBS at a density of 10^6 cells/mL. Cells were placed on 35 mm dishes with coverglass bottoms and allowed to settle for 10 minutes; other than this settling they remained suspended during imaging. Hoechst 33342 (H3570, Life Technologies, Australia) 5 µg/ml was used for nucleus staining. Cell Mask Deep Red (C10046, Life Technologies Australia) 5 mg/ml was used for membrane staining. For each group of control and treated cells. Spectral images were collected at 405-nm excitation wavelength and detected in 430-470 nm emission range for Hoechst and at 633 nm excitation wavelength and emission at 650-690 nm for Cell Mask Deep Red. FITC and Dragon Green was detected using 488 nm excitation and 520-560 nm

emission range. Phase contrast images were collected for all groups. All samples were imaged at the same parameters of pinhole aperture and detector voltage.

Cell biotinylation and conjugation of neutravidin

Two T75 cm^2 flasks of 90-95% confluent cells (10^7 cells each flask) were prepared. Cells were harvested at a density of 8.5-10 × 10^6 cells/mL and a volume of 1 mL of cell solution was suspended in 5 mL media. The cells were labelled with biotin using the protocol described in Pierce® Cell Surface Protein Isolation Kit (CAS No. PIE89881, Thermo Fisher Scientific). Specifically, the media was removed and cells were washed twice with 8 mL of ice-cold PBS per flask. The PBS was removed within 5 s. After that, 10 mL of the ice-cold biotin solution (1 mg mL^{-1}) was added to each flask, which was then placed on a rocking platform to gently agitate for 30 minutes at 4°C to ensure even coverage of the cells with the biotinylation solution. 500 µL of quenching solution was added to each flask to quench the reaction and the flask was gently tipped back and forth to ensure even coverage of the solution. The cells were gently scraped using the cell lifter into solution. The contents of two flasks were transferred to a single 50 mL conical tube. Both flasks were finally rinsed with a single 10 mL volume of tris-buffered saline (TBS) pH 7.2 and then the rinse volume was added to the transferred cells in the conical tube. Cells were centrifuged at 200 rcf for 6 min and supernatant was discarded. Cells were re-dispersed in PBS at cell density of ~10^6 cells/mL.

The 4 µg neutravidin (Thermo Fisher Catalog number 31000) was added to each 1 mL of cell solution. To the cell pellet, 5 mL TBS was added and cells were gently pipetted up and down twice with a serological pipette, which were centrifuged at 200 rcf for 6 min and supernatant was discarded. The obtained cells were collected for the antibody attachment as described below.

OnCELISA labelling

The biotinylated cells with neutravidin added (10^6 cells) were resuspended in 1 mL of gelatinous medium (25% gelatin) containing 2 µg biotinylated mouse anti-IL-6 goat IgG4 (CAS No. BAF406 from R&D Systems) (0.5 mg mL^{-1} in 1 × PBS) for 2 h at 37 ^oC. Then the cells were washed twice in 25 mL PBS, pH 7 and the cell pellets were collected. Cells were resuspended in 1 mL of 37^oC gelatinous medium (25% gelatin). After having been exposed to cytokines (either spiked into the medium, or secreted upon cell stimulation DG SPIO_IL-6_Ab

(10 μL per each 1 mL of cell solution at 10^6 cells/mL) was added to the medium and allowed to bind. After 1 h, the cells were washed 2 times with 1 × PBS.

Calibration curve of OnCELISA for detection of cytokines

The BV2 cells were prepared with the capture antibody conjugated on the cell surface according to the above OnCELISA protocol. Cell pellets were collected and cells were divided into 1 mL tubes at cell density of 10^6 cells/mL. Recombinant mouse IL-6 (CAS No.: 406-ML) at different concentration (from 0 to 1000 pg/mL) was externally added into individual tubes. Each concentration was spiked into 3 tubes to provide triplicate assay readings at that concentration. After waiting for 10 min, the cells from each tube were washed 2 times with 1 × PBS, and dispersed in 1 mL medium. The OnCELISA protocol was then completed, by adding DGSPIO_IL-6_Ab (10 μL) to the medium and this labelled detection antibody was then allowed to bind for 1 h. After this time, the cells were washed 2 times with 1 x PBS. Finally, the cells from each tube were dispersed in 1 mL PBS for fluorescence reading by using Fluorolog Tau-3 from Jobin-Yvon-Horiba. The results are plotted in Figure S3 b as average and standard deviation of the triplicate readings at each concentration.

Sample preparation for ELISA

BD OptEIA^{Tm} Mouse IL-6 ELISA kit (CAS No. 550950, BD Bioscience, Australia) was used to measure the concentration of IL-6 secreted by cells after LPS stimulation. For preparation of IL-6 samples, the biotinylated cells with the density of 10^6 /mL were suspended in 1 mL of warm medium containing 100 ng mL^{-1} LPS from Escherichia coli 026:B6 (Sigma Aldrich, Australia) to secrete IL-6 for 0 h, 2 h, 4 h, 6 h, 8 h, and 20 h, respectively. Supernatants from cells were collected in duplicate, and analysis was performed according to the manufacturer's instructions. For control measurements, IL-6 samples secreted by the original cells (without any functionalisation) were also prepared. All tests were performed using Nunc MaxiSorp 96 well plates, supplied with the ELISA Kit. BMG FLUOstar Galaxy Microplate Reader was used to measure absorbance at 450 nm. Optical density was also measured at 570 nm for wavelength correction. Results are analyzed and reported as means ± standard deviation.

Magnetic sorting

A magnetic sorter PickPen (Luoyang Huier Nani Science and Technology Co. LTD) was used to sort the cells labelled with fluorescent magnetic particles. In order to demonstrate

magnetic sorting, two suspensions of cells labeled with particles were prepared. One suspension was washed by using a normal protocol by centrifugation and cell strainer separation. Another suspension was washed by the application of magnetic pen in cell suspension for 1 min, and then the tip of magnetic pen was released in a clean tube and washed with PBS. Then the washed cells were collected for confocal imaging.

Size and zeta potential measurement

The hydrodynamic size and the zeta potential of the magnetic nanoparticles before and after conjugations with the antibody were determined using Zeta Sizer Nano Series Nano-ZS (Malvern Instrument, UK).

Fluorescent *in situ* hybridization

Primers for fluorescent *in situ* hybridization (FISH) were designed using Primer3 (NCBI Nucleotide), spanning exon-exon junctions and including the functional protein coding region of the mouse IL6 mRNA sequence (NCBI accession NM 031168). The forward primer includes the SP6 promoter sequence (ATTTAGGTGACACTATAGAAG) at the 5' end while the reverse primer includes the T7 promoter sequence (TAATACGACTCACTATAGGGAGA) at the 5' end. IL6 F Primer with SP6 at 5' end: 5'ATTTAGGTGACACTATAGAAG-GGGACTGATGCTGGTGACAA 3'; R Primer with T7 at 5' end: TAATACGACTCACTATAGGGAGA-TAACGCACTAGGTTTGCCGA. Standard PCR was performed for IL6 mRNA using mouse BV2 cell cDNA, the resulting PCR product transcribed was from bp 76 to bp 674 (599bp total length) of the mouse IL6 mRNA sequence. In each 25 µL reaction tube: 12.5 µL AmpliTaq Gold® 360 Master Mix (Life Technologies), 1 µL Forward Primer, 1 µL Reverse Primer, 1 µg cDNA, 9.5 µL RNAse/DNAse free water. Additionally, no template control and no primer control reactions were run in parallel for comparison to IL6 PCR product. Tubes were held at 95°C for 10 min to activate the Taq enzyme followed by 40 cycle repeats of 95°C 30 sec (denaturation), 60 °C 30 sec (annealing), 72 °C 60 sec (extension) and a final extension at 72 °C for 7 min. PCR products were purified using a column extraction kit according to the manufacturer protocol (PureLink® PCR Purification Kit; Life Technologies). PCR products were then run on a 2% TAE gel containing SYBR Gold and photographed on a Genesnap gel doc (Syngene) to confirm appropriate molecular weight and the absence of primer dimer.

Purified IL-6 PCR product was *in vitro* transcribed to complimentary RNA strands using the MEGAscript® T7 Transcription Kit (AM1334; Life Technologies) according to the manufacturer protocol using 200 ng of PCR product from each gene. Biotin-UTP and biotin-CTP were incorporated into the IL6 cRNA probe. Sense IL6 control strands were *in vitro* transcribed using the MEGAscript® SP6 Transcription Kit (AM1330; Life Technologies) according to the manufacturer protocol. All RNA probes were purified with $LiCl_2$ solution, washed with EtOH, air dried and reconstituted in 40 µL of RNAse/DNAse free water. RNA probe quality and quantity was then assessed using a Nanodrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA probes were run on a denaturing formaldehyde gel with MOPS buffer using electrophoresis to confirm molecular weight and specificity.

BV2 cells were treated with LPS (1.0 µg mL^{-1} concentration) in 2 ml of their normal DMEM on coverslips in 6 well plates. LPS in DMEM was removed immediately and replaced with PBS pH 7.2 for 0 h LPS treatments. Cells inside the manufactured microchip, or on coverslips were first washed in PBS pH 7.2 and fixed using 4% formaldehyde for 10 min and then again washed with PBS. They were permeabilised with 70% EtOH for 30 min at 4 ^{O}C then washed with PBT solution (PBS + 0.01% Tween-20). Pre-hybridization buffer (50% formamide, 5 × SSC pH 7.0, 250 µg mL^{-1} herring sperm DNA, 5% dextran sulfate, 1X Denhardt's solution, 0.1% Tween-20) was added to cells with 1000ng of cRNA probe and incubated for ~16hrs in an incubator at 37 ^{O}C. Cells were washed 3x in 2X SSC buffer (sodium citrate/NaCl solution) followed by 3 × washes in PBT solution. Samples were incubated for 1hr at room temp (~22 ^{O}C) with 2.5 µg of streptavidin-Alexafluor647 or streptavidin-Alexafluor555 (Molecular Probes, Life Technologies). Cells were then washed 3 × 5 minutes in PBT solution on an orbital shaker. Cells stained on coverslips or microchips were mounted onto slides in Prolong Gold Antifade Media with DAPI dye (P 36931, Life Technologies) and photographed with wide field (Zeiss Axioimager Z1) or confocal microscopy (Leica Microsystems SP2).

ImageJ (http://rsb.info.nih.gov/ij) was used for FISH analysis on slides. Data were calculated at a threshold of 10% maximum brightness intensity. All data is presented as mean ± SEM. Data was compiled and graphed using Graphpad Prism software (Version 6.07). Student's t-test was used for statistical analysis between treatments where $p<0.05$ was considered significant.

Fabrication of SU-8 molds and PDMS chips with single cell wells

The procedure was based on Reference [1]. The entire array of single cell wells was designed to be 40.00 mm long and 0.90 mm wide. It had a rectangular array of 25 μm diameter pillars also 25 μm apart, 900 pillars along the array, 18 pillars across the array. Using this layout pattern, 20-μm thick SU-8 master molds were created on 3" wafers using standard photolithography processes. An intermediate glass-supported PDMS (Sylgard 184 prepolymer) mold was created from the SU-8 master. This intermediate was then treated with a 430 mtorr oxygen plasma and silanized by soaking in isopropyl alcohol containing 1% OTS (octadecyltrichlorosilane) for 15 min. The same PDMS prepolymer was then poured onto the intermediate mould and cured at 65℃. The final PDMS substrate was peeled off. It had 25 μm diameter holes and 25 μm hole-to-hole gaps, and it was abricated with depth of each hole of 20 μm. Furthermore, the PDMS substrate was diced to create single rectangular microchip units with 27 holes in one direction) and 18 holes in the perpendicular direction. The 2.5 mL microcentrifuge tubes were filled with 1.5 mL of PDMS, which was cured. The diced PDMS pieces containing wells were placed in the tubes and glued into place with the wells facing up. These tubes were then treated with OTS (as above) to reduce non-specific adhesion. Following a rinsing with isopropyl alcohol the tubes containing single-cell-wells were ready to use.

Placing single cells in wells

The microchip was mounted perpendicularly inside a 1.5 mL in an Eppendorf tube. The cell solution 50 μL at cell density of 2×10^6/mL was placed in the tube and centrifuged for 5 minutes at 1500 rpm. This made it possible for cells to be placed individually in the wells. After the application of OnCELISA in single cells in wells, the mounted microchip was taken out from the tube and placed on a petri dish for confocal microscopy imaging.

Animal study protocol

Mice were bred at the Heart Research Institute Sydney, Australia. Protocols were approved by the Sydney Local Health District Animal Ethics Committee (2014-014) (Sydney, Australia). Six-week-old atherosclerosis-prone male apolipoprotein $E^{-/-}$ mice were fed standard chow for 16 weeks to develop atherosclerotic plaque in their aortic arches and descending thoracic aortae. Two hours prior to euthanasia by cardiac exsanguination, mice were injected with LPS (100 μg/mouse) or PBS i.p. Aortae were excised. Single cell suspensions were prepared from the

aortic tissue using an enzymatic digestion containing Collagenase I and Collagenase IX (both from Sigma Aldrich) and then passage through a cell strainer (70 µm). Cells were subjected to the OnCELISA using antibodies raised against mice (anti-IL-6 monoclonal antibody). Cells were subjected to flow cytometry on a BD FACSVerse (BD Biosciences) and the data was analysed using FlowJo® software.

Statistics

One-tailed t-test was performed for the investigated groups of animals in the atherosclerosis study. The test was applied by using software Prism. The level of statistical significance was set at $p < 0.05$

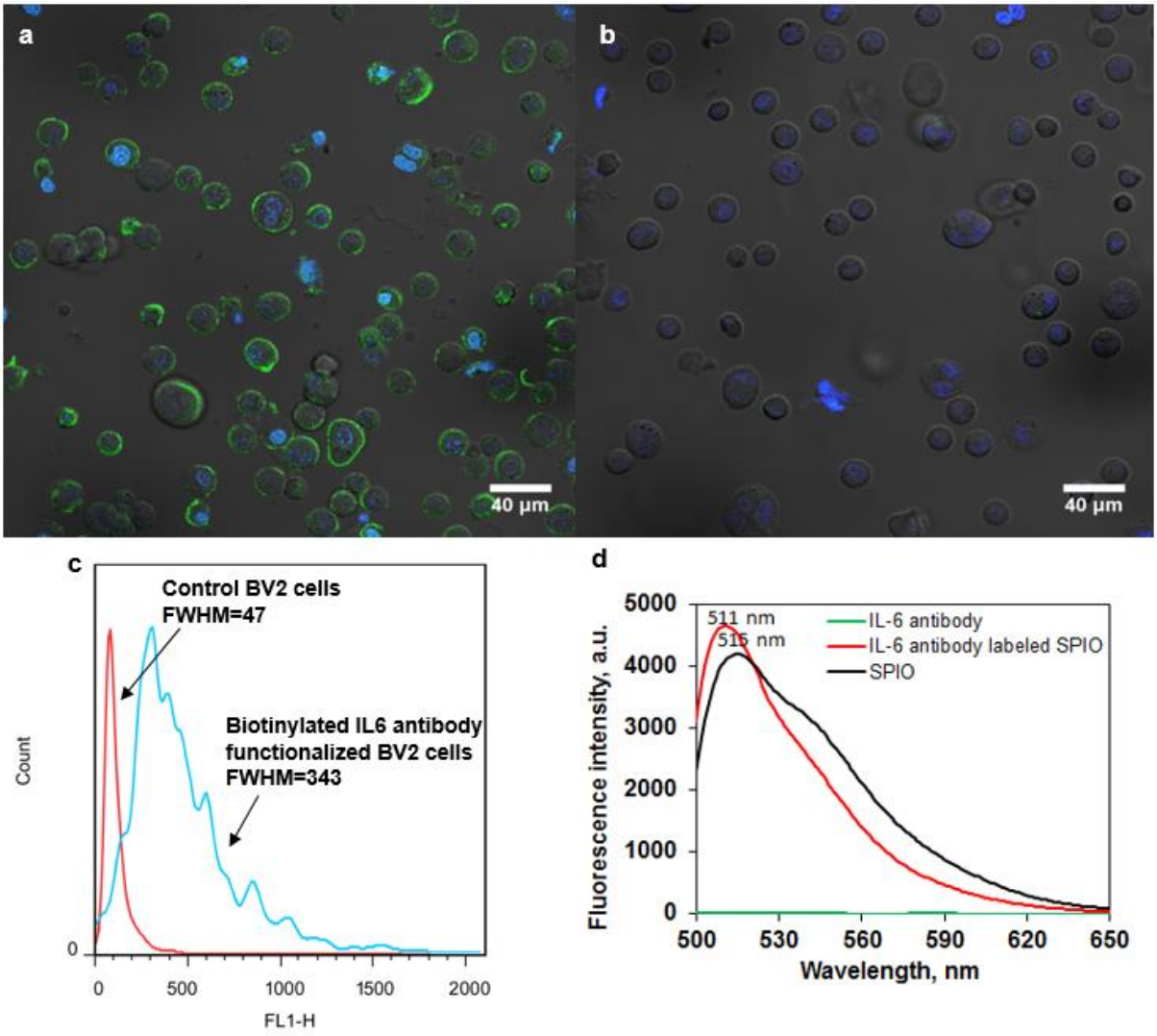

Figure S1. Demonstration of the localization of the capture antibody and detection when combined with fluorescent magnetic beads (SPIO). a) Confocal image of the IL-6 capture antibody in BV2 cells after treatment with FITC labeled secondary antibody. Image has been combined with a DIC image of the same field of view. b) Confocal image of BV2 cells that have not been incubated with the capture antibody, after treatment with FITC labeled secondary antibody (negative control). Image has been combined with a DIC image of the same field of view. c) Flow cytometry histograms for control BV2 cells (without the capture antibody, red line) and IL-6 capture antibody-modified BV2 cells after treatment with FITC labeled secondary (blue line). d) The fluorescence spectra for IL-6 only (green line), IL-6 + SPIO (red line) and SPIO only (black line). The Dragon Green magnetic particles (SPIO), IL-6_Ab, + SPIO_IL-6_Ab were excited at 480 nm. Related to Figure 1.

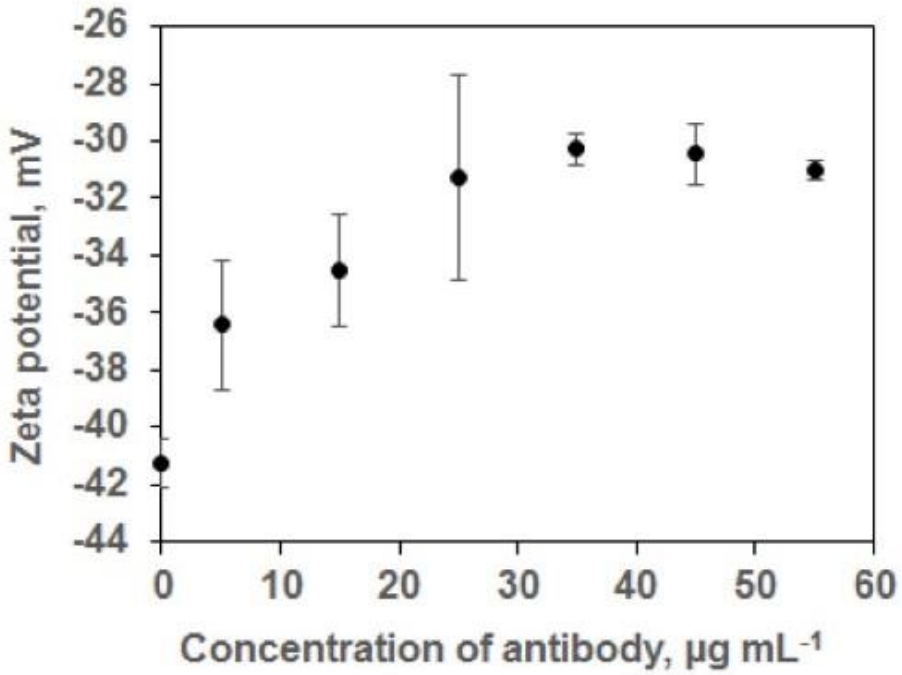

Figure S2. Confirmation of antibody attachment to magnetic nanoparticles. The zeta potential for magnetic nanoparticles before (0 µg/mL) and after conjugation of IL-6 antibodies at different concentrations. The zeta potential decreases with the increase in IL-6 antibody concentration reaching a plateau at 8 µg mL^{-1}. Related to Figure 1.

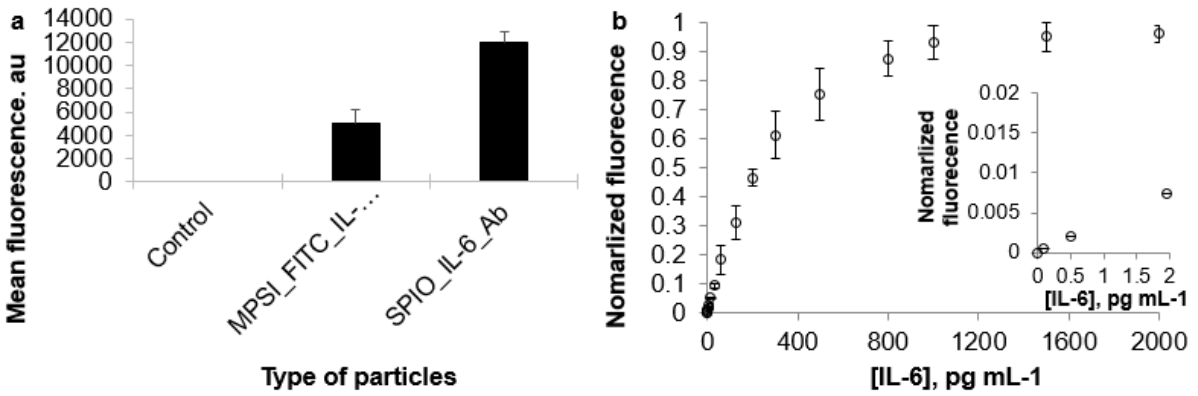

Figure S3. a) The affinity of the IL-6 antibodies conjugated to DG SPIO_IL-6_Ab and to MPSi_FITC_IL-6_Ab determined by the fluorescence plate reader. In this experiment IL-6 (200 pg mL^{-1}) was added to 96 well plate followed by adding MPSi_FITC_IL-6_Ab or DG SPIO_IL-6_Ab, and finally, the wells were washed 5 times with PBS. The ELISA Plate reader (BMG FLUOstar Galaxy-Multi-functional Microplate Reader) was used to detect fluorescence (excitation at 492 nm). The DG SPIO_IL-6_Ab particles show a stronger fluorescence signal than the MPSi_FITC_IL-6_Ab. b) Sensitivity and linear range of the OnCELISA assay (functionalized cells with the capture antibody on the surface incubated with DG SPIO_IL-6_Ab particles as the detection antibody). Based on the calibration curve of IL-6 shown in b) the low detection limit is 0.1 pg mL^{-1} and the linear range of the assay is 0.1-1000 pg mL^{-1}. Note that this low detection limit was obtained with fluorimetry readout, and the corresponding low detection limit using microscopy is different (higher). Related to Figure 1.

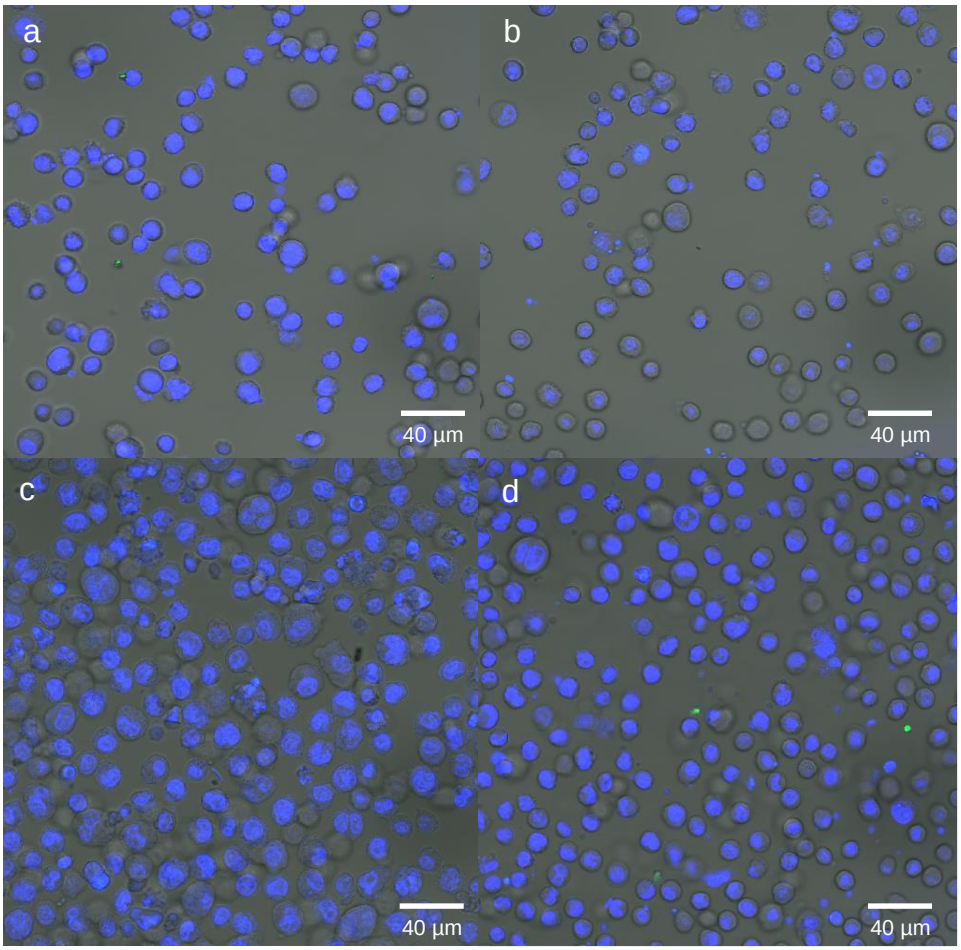

Figure S4. Systematic control experiments confirming the importance of each constituent of the OnCELISA. Confocal laser scanning microscopy images combined with DIC images for control samples (blue is Hoechst nucleus staining, DG SPIO nanoparticles are shown in green). The following controls were carried out: a) Biotinylated BV2 cells were treated with DG SPIO_IL-6_Ab, without neutravidin. The results demonstrate only very low (< 5%) non-specific adsorption of nanoparticles on biotinylated cell surfaces or nanoparticle uptake. b) Biotinylated BV2 cells were treated with neutravidin, and then DG SPIO_IL-6_Ab after LPS stimulation to verify that the presence of biotinylated anti_IL-6_Ab is vital to the function of the prepared affinity surface. c) Biotinylated BV2 cells were treated with neutravidin, a biotinylated mismatched antibody (anti IL-1β_Ab), and then with DG SPIO_IL-6_Ab after LPS stimulation. d) Biotinylated BV2 cells were treated with neutravidin, biotinylated anti_IL-6_Ab, and then DG SPIO with a mismatched anti-chicken Ab after LPS stimulation (labelled with Alexa Fluor® 488 conjugate) to establish the selectivity of the prepared capture surfaces. Related to Figure 1.

Table S1. Details for all control experiments. Related to Figure 4.

	Components of OnCELISA assay					
	OnCELISA results	**Biotinylation**	**Neutravidin**	**Biotinylated anti IL-6 Ab**	**IL-6**	**DG SPIO_IL-6_Ab**
Control 1	Negative	Yes	No	No	Yes	Yes
Control 2	Negative	Yes	Yes	No	Yes	Yes
Control 3	Negative	Yes	Yes	Yes, but replaced by DG SPIO_IL-1β_Ab	Yes	Yes
Control 4	Negative	Yes	Yes	Yes	Yes	Yes, but replaced by DG SPIO_anti-chicken_Ab

Table S1 details for control experiments to validate the components of the OnCELISA assay. The results show that there is negligible non-specific adsorption of nanoparticles on biotinylated cells. In addition, almost no labelling was observed when the biotinylated anti-IL-6 capture antibody was not modified on the cell surfaces, suggesting that the capture antibody is very important to the function of the affinity surface. No fluorescence was observed after functionalized cells were treated with the fluorescent detection antibody anti-chicken IgG which has no affinity to IL-6, suggesting that the capture surface is selective to IL-6. All these controls suggest that the cells labelling is due to the affinity between IL-6 antibody and the IL-6 secreted by cells, and not to non-specific adsorption or uptake.

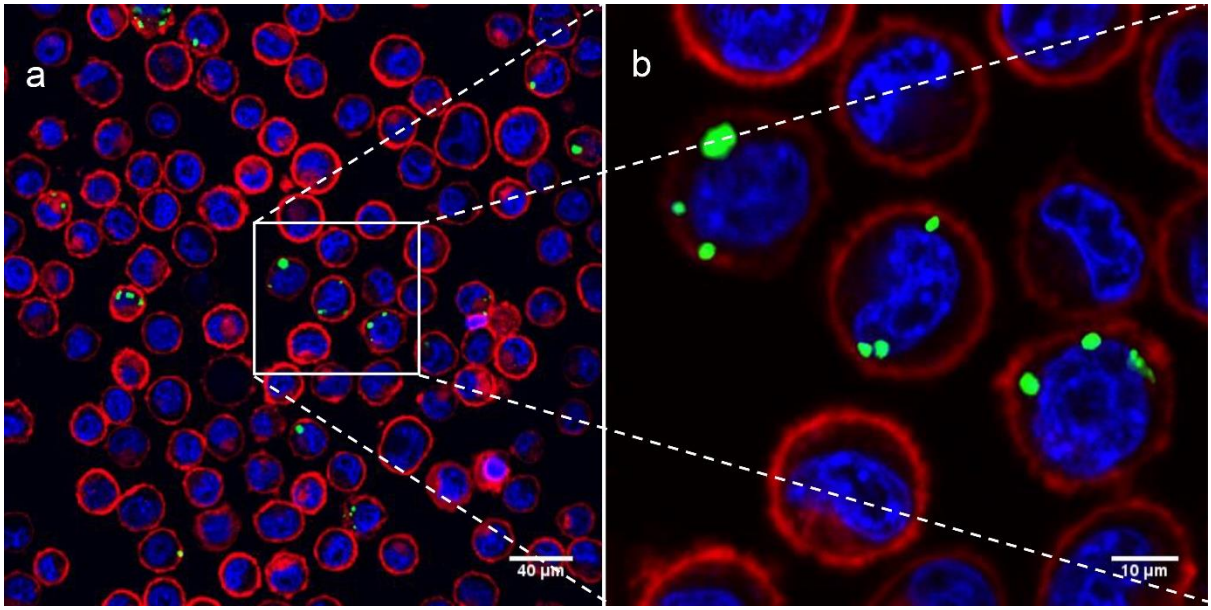

Figure S5. Confocal images for BV2 cells after treatment with OnCELISA (green) combined with nuclear (blue) and membrane (red) staining (a) at lower magnification; (b) at higher magnification. Related to Figure 1.

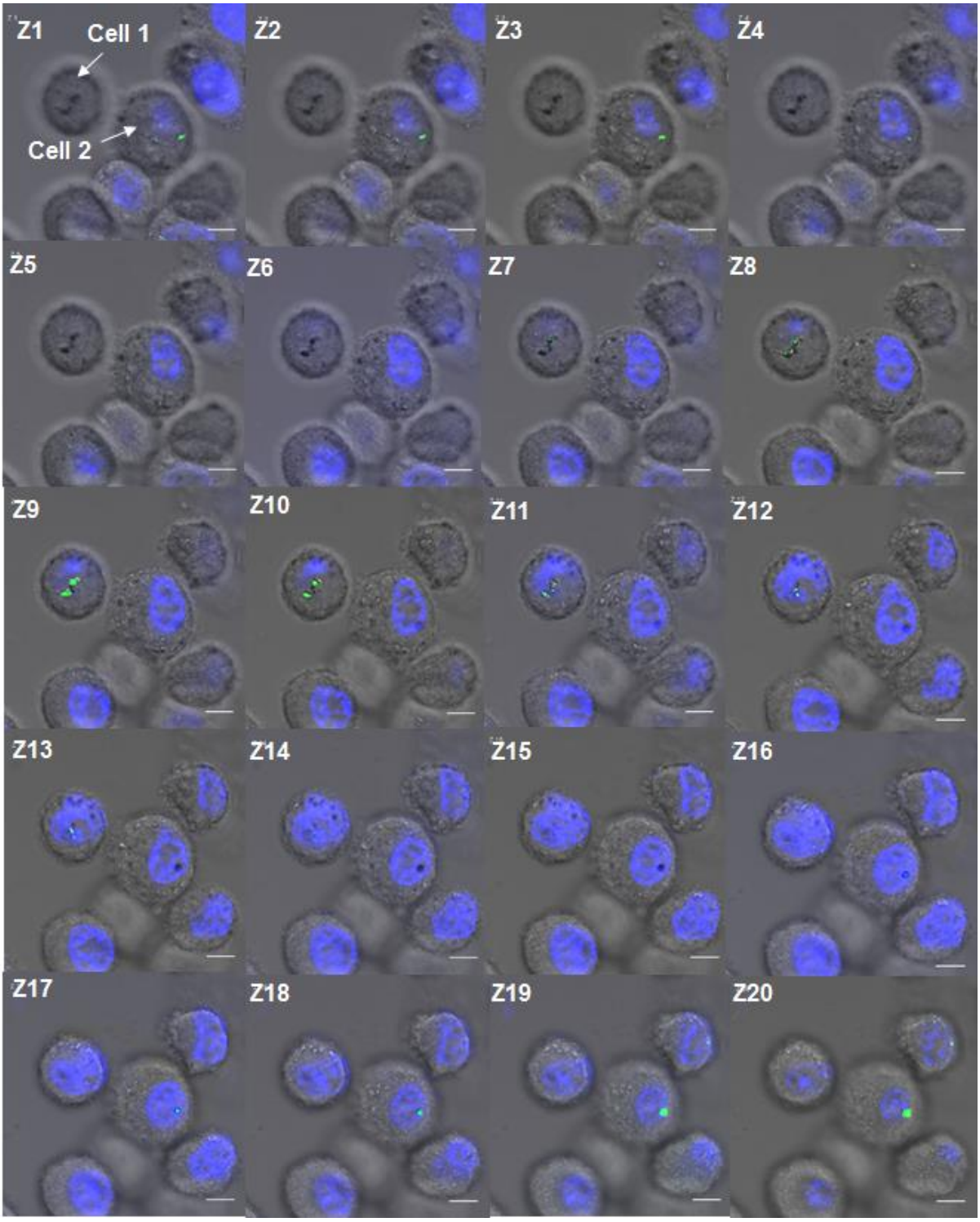

Figure S6. Consecutive Z-stack images showing the location of of OnCELISA labelling. In this figure confocal images have been combined with DIC images of the same field of view. Z-separation of individual images is 1.05 um. Upon inspection of consecutive images Z-1 through to Z-9 we can see Cell 1 slowly coming into focus. The image plane significantly intersects the cell nucleus on images Z-11 to Z-19, with maximum cross-section around image Z-15, marking the approximate center of the cell. In contrast, the OnCELISA labeling on Cell

1 is brightest in the Image Z-9, consistent with labeling on the top or bottom of the cell. Similar effect is seen in Cell 2 where OnCELISA labeling is observed at the beginning and at the end of the presented Z-stack, where the cell cross-section seems to be out-of focus, but the nucleus seems the biggest around the middle of the stack. Scale bar = 10 μm. Related to Figure 1.

Vesicular model of cytokine release

The below section is to formulate a mathematical model the process of cytokine release and determine its impact on chemical kinetics of the OnCELISA immunoassay. We intend to establish whether OnCELISA on an individual cell detects its own cytokines, secreted from that particular cell or whether it detects cytokines secreted from adjacent cells. To this aim, we calculate the fraction of the cell-bound Abs in the OnCELISA assay that are able to detect its own molecules (akin to autocrine detection), secreted by this particular cell and self-captured. We consider a vesicular release mechanism of cytokines[2], which produces short bursts of locally high cytokine concentrations.

Cytokines are released from immune cells via diverse pathways, some of which involve secretory vesicles [2]. In our model A we assume that the IL-6 is released from spherical secretory vesicles with radius $r_{vesicle}$ that are below the optical diffraction limit ($r_{vesicle}$ =0.1 um) and that the concentration of IL-6 in such vesicles, $N_{vesicle}$ is high ($N_{vesicle}$= 10 mM, see **Table S2** for summary of all numerical values used in these calculations). Once cytokines are released from the vesicle, they form a hemispherical cloud of molecules with an average concentration $C_{released}(t)$. In the case of diffusion in free space away from a molecular source, the average square distance of molecules from the source over time t is Dt where *D* is the diffusion constant. Therefore, the radius of the hemispherical cloud is $R = \sqrt{Dt}$. In our simplified model we assume that the released molecules form a uniform density hemispherical cloud of this radius. The background cytokines produced by other cells are also present, with a uniform low concentration, C_b. The total concentration of cytokines available for binding in the cytokine cloud is $C(t) = C_{released}(t) + C_b$, and outside it is C_b. C_b is generally small compared to $C_{released}(t)$ on the timescale of single seconds considered here. In this case the released average cytokine density in the proximity of the cell surface can be approximated by:

$$C(t) = \frac{1}{2} N_{vesicle} \left(\frac{r}{(Dt)^{1/2}} \right)^3 \qquad /1\text{-}1/$$

Estimates of relevant physical constants

Estimate of diffusion constant of IL-6 in water and in gelatin solutions. In experimental protocols for cell-surface assays, gelatin may be used to slow down diffusion[3]. In this section we estimate the diffusion constant of IL-6 in the media containing water, 25% gelatin, and 40% gelatin respectively. To estimate this diffusion constant we used the approach in Reference[4]. IL-6 is assumed to be a spherical molecule [4]. The diffusion of spherical molecules is given by the Stokes-Einstein equation:

$$D = \frac{k_B T}{6\pi\eta r} \qquad /1\text{-}2/$$

where T is the absolute temperature in Kelvins, r is the Stokes radius of the spherical molecule and η is the viscosity of the solvent at the experiment temperature, in [Pa s], k_B is the Boltzmann constant. The Stokes radius is a function of molecular weight and between 4 kDa and 40 kDa and it varies from 2 nm to 6.5 nm [4]. IL-6 has 24 kDa on average with 4 close forms with 22 to 26 kDa, therefore we have taken r = 5 nm to be the Stokes radius of IL-6.

Viscosity values in gelatin solutions. Here we estimate the viscosity of gelatin solutions, featuring in the Stokes equation. The intrinsic viscosity $[\eta]$ defined as the ratio of specific viscosity η_{sp} to *c*, the concentration of polymer in grams per 100 cm^3 in the limit of small *c* is between 40 and 80 cm^3/g,[5] and is usually equal to the ratio of $[\eta] = \frac{\eta_{sp}}{c}$ [5]. The specific viscosity is obtained from relative viscosity as η_r as

$$\eta_{sp} = \eta_r - 1 \qquad /1\text{-}3/$$

while relative viscosity is the ratio of the viscosity of the solution to that of the solvent.

Using the value of $[\eta]$ = 50 cm^3/g and the concentration of gelatin, *c* = 25 g/100 cm^3 (25 % gelatin) we get

$\eta_{sp} = [\eta]c = 12.5$, $\eta_r = 13.5$. Using the value of viscosity of water of 0.8 mPa s [6] we obtain the viscosity of 25% gelatin η =10.8 mPa s. We remind that Pa = N/m^2.

For *c* = 40 g/100 cm^3 (40% gelatin) we obtain η = 16 mPa s. Substituting into Equation /1-2/ we get $D = 4 * 10^{-8}$ cm ^{2}/s for 25% gelatin and $D = 2.5 * 10^{-8}$ cm^2/s for 40% gelatin. These values can be compared with the diffusion constant for Il-6 in water of $2.7 * 10^{-7}$ cm^2/s [7], and for GFP in water of $8.7 * 10^{-7}$ cm^2/s reported in Reference.[4]

Table S2. Values of parameters used in the mathematical modeling.

Quantity	Value	Reference
v_{sample}	1 cm^3	n/a
n_{cells}	10^6	n/a
ρ	10 μm = 10^{-3} cm	n/a
$N_{vesicle}$	10 mM	n/a
$r_{vesicle}$	0.1 um	n/a
D	$2.5 * 10^{-8}$ cm^2/s	Our estimates, see above, for 40% gelatin
∇T	$0.25 * 10^3$s	Francis, K.; Palsson, B. O. *Proceedings of the National Academy of Sciences* **1997**, *94*, 12258.

Analysis of chemical kinetics. The interaction of cytokines released from the vesicles is further described in terms of a chemical reaction framework through capture antibody and cytokine concentrations, kinetic rates, and binding affinity. In respect to binding kinetics, we adapt here the model presented in Reference [8]

IL-6 binding to antibodies on the cell surface is described in terms of the effective on-rate K_{on} and off-rate k_{off}. The number of antibody-IL-6 bonds per unit area, $N(t)$ satisfies the relationship [8]:

$$\frac{dN(t)}{dt} = K_{on}[N_{max} - N(t)] - k_{off}N(t) \qquad /1\text{-}4/$$

Here, N_{max} is the maximum number of bonds given by the antibody density per unit cell area. The on-rate K_{on} is a lumped kinetic constant $K_{on} = k_{on} * C(t)$ where k_{on} is the on-rate per antibody molecule (in units of $(Ms)^{-1}$). The equation has the solution:

$$N(t) = \frac{K_{on}N_{max}}{K_{on}+k_{off}}\{1 - exp[-(K_{on} + k_{off})]\}, \qquad /1\text{-}5/$$

or substituting for K_{on}:

$$N(t) = \frac{C(t)k_{on}\,N_{max}}{C(t)k_{on}+k_{off}}\{1 - exp[-(C(t)k_{on} + k_{off})]\} \qquad /1\text{-}6/$$

Numerical estimates of binding kinetics. We now estimate the bond density. To this aim, we note that the antibodies used here had the affinity constant $\frac{k_{on}}{k_{off}}$ in the order of 10^{11} M^{-1} [9] . The parameter k_{off} is taken to be in the range of 10^{-5} s^{-1} to 10^{-4} s^{-1} [10]. Correspondingly, k_{on} is in the range of 10^6 -10^7 $(Ms)^{-1}$. In the limit of large cytokine concetrations discussed here the contribution from k_{off} is negligible and $N(t)$ can be approximated by:

$$N(t) \approx N_{max}\{1 - exp[-(C(t)k_{on})]\} \qquad /1\text{-}7/$$

The bond density increases exponentially with a time constant τ such that $\tau^{-1} = C(\tau)k_{on}$. In particular, substantial, 63% (63%=$\left[1 - \frac{1}{e}\right]$) antibody binding is achieved after time τ and higher percentage binding is achieved for longer times. We now estimate this time constant for our estimated cytokine concentration..

$$\tau = \frac{1}{C(\tau)k_{on}} = \frac{2}{k_{on}N_{vesicle}} \left(\frac{(D\tau)^{1/2}}{r_{vesicle}}\right)^3, \qquad /1\text{-}8/$$

Or, solving for τ

$$\tau = \frac{(k_{on}N_{vesicle})^2 {r_{vesicle}}^6}{4D^3} \qquad /1\text{-}9/$$

Substituting the values for 25% gelatin and taking k_{on} = 10^6 - 10^7 $(Ms)^{-1}$, $N_{vesicle}$=10 mM and vesicle radius $r_{vesicle}$=0.1 μm and *D*= 4 $\mu m^2/s$ we obtain: $\tau_{0.25} =$ 0.4 s and this >63% binding will take place in the region of radius $(D\tau)^{1/2}$=1.25 μm. For 40% gelatine we obtain $\tau_{0.4} =$ 1.59 s and this >63% binding will take place in the region of radius $(D\tau)^{1/2}$=1.99 μm.

Conclusions: Can OnCELISA detect cytokines from other, non-secreting cells by the virtue of their proximity to a secreting cell? With the assumptions of our model we now answer the question whether there is cross-labeling from a secreting cell to other, potentially non-secreting cells. Our calculations indicate that the cytokines from any adjacent cell whose secreting vesicle is closer to the selected cell than the diffusion distance of 1.25 μm (25% gelatin) and 2 μm (40% gelatin) will be able to attach to the antibodies at the adjacent cell and saturate the bonds to 63% - which means that the adjacent cell will be labelled despite being non-secreting. However, this is a relatively rare occurrence and the labelling of the non-secreting cell touching only 1 secreting cell will remain very low. Simple geometry considerations indicate that two spherical cells 10 um diameter and touching one another have

a small percentage (4.8%) of their area closer than 2 μm to one another (and correspondingly less for 1.25 μm diffusion distance). Labelling of the non-secreting cell would require the appearance of the secreting vesicle in the secreting region immediately adjacent to the non-secreting cell, and the number of such vesicles would be proportional to the area ratio (that is scaled down by a factor of 4.8%). This means that even if the vesicle would eventually be present within the appropriate area, the non-secreting cell would only be able to be labelled in the small area representing 4.8% of the cell area. The situation becomes less clear-cut in the case when there is a larger fraction of highly secreting cells. In the case of a single non-secreting cell that is surrounded by closely packed secreting cells in the hcp lattice where each cell has 12 neighbors, over 50% of the area of the non-secreting cells could, in principle be labelled. This may set a limit on the purity of cell subpopulations selected by using OnCELISA, but it could be partially moderated by setting comparatively high gating limits in flow cytometry sorting.

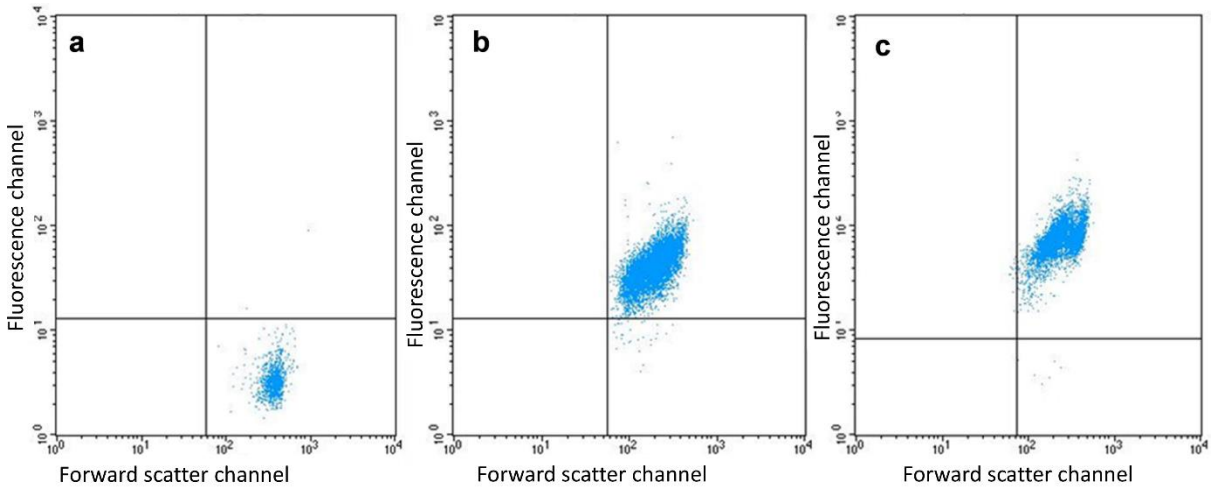

Figure S7. The progeny cells of biotinylated BV2 cells are also biotinylated. (a) control BV2 cells; (b) biotinylated BV2 cells; (c) progeny of biotinylated BV2 cells. The cells were stained with FITC-avidin before flow cytometry measurement. Related to Figure 4.

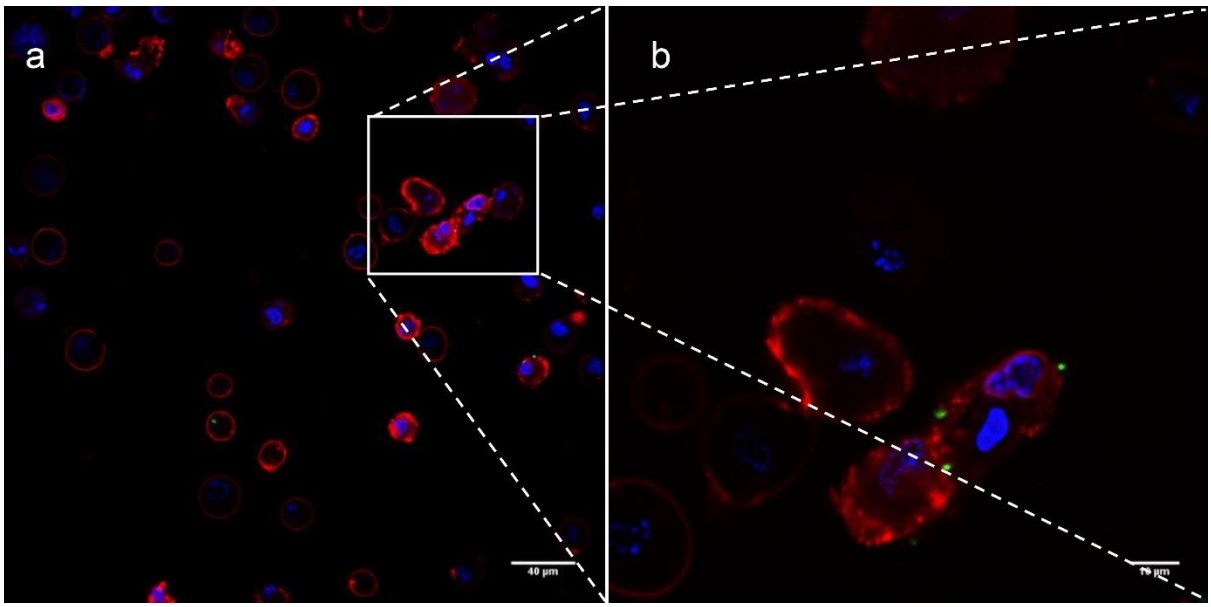

Figure S8. OnCELISA applied to mesenchymal stem cells (MSCs). (a-b) Confocal laser scanning microscopy images for mesenchymal stem cells (MSCs) which have been labeled with DG SPIO_IL-6_Ab at different magnifications. Related to Figure 1.

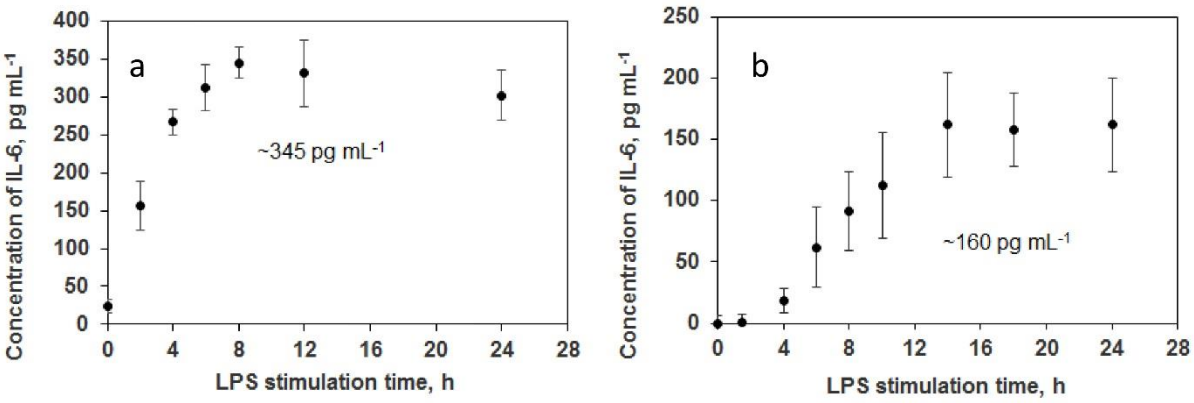

Figure S9. ELISA measurements of IL-6 secreted from RAW cells (a) and MSCs (b). Related to Figure 1.

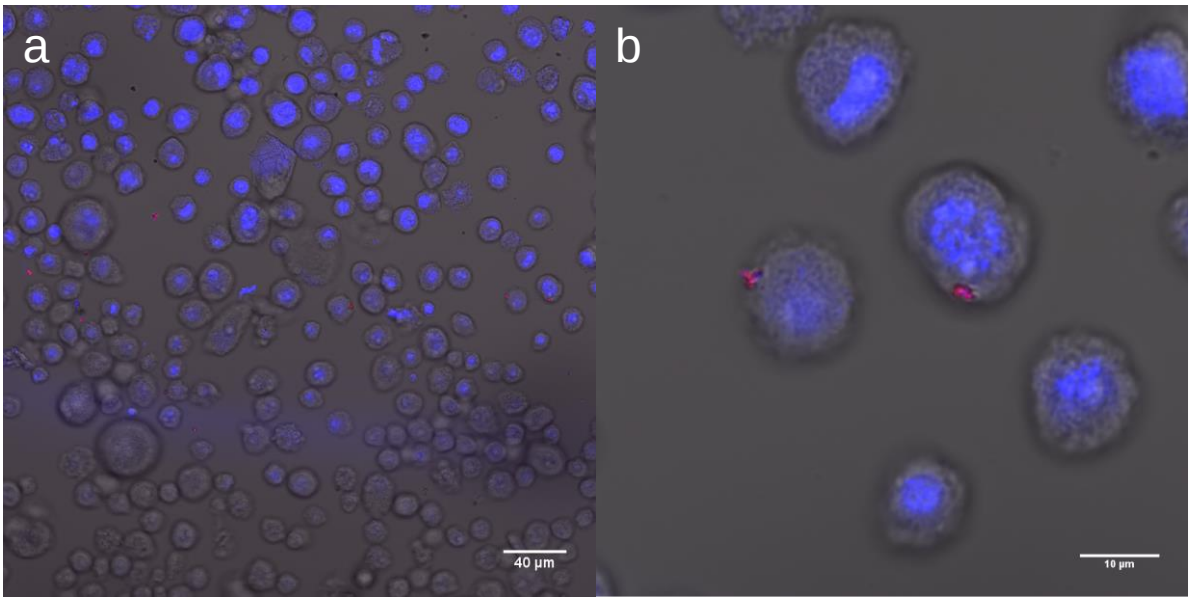

Figure S10. OnCELISA can detect the secretion of IL-1β. Confocal laser scanning microscopy images combined with DIC images for functionalized cells with 8 h LPS stimulation after treatment with Flush Red SPIO_IL-1β_Ab at different magnifications. Related to Figure 1.

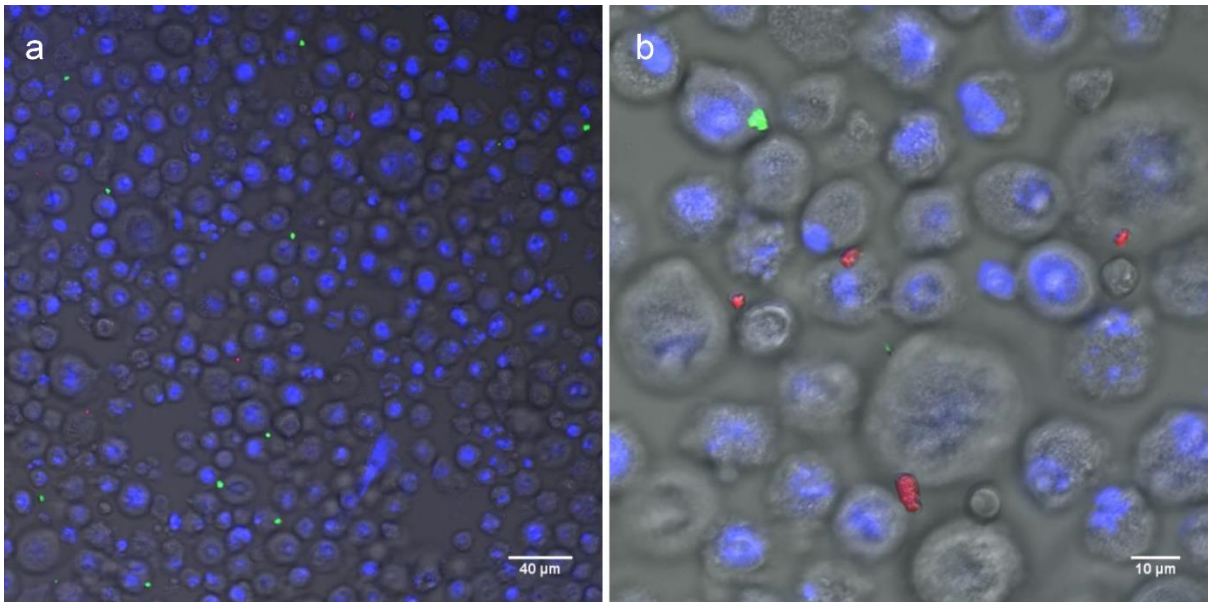

Figure S11. OnCELISA can be simultaneously used with two different colour nanoparticles and two cytokines, IL-6 and IL-1β. Confocal laser scanning microscopy images have been combined with DIC images of the same field of view. Images show functionalized cells with 8 h LPS stimulation after treatment with DG SPIO_IL-6_Ab and flush red SPIO_IL-1β_Ab at different magnifications. Related to Figure 1.

References

1. Huang, L., Chen, Y., Chen, Y.F. & Wu, H.K. Centrifugation-Assisted Single-Cell Trapping in a Truncated Cone-Shaped Microwell Array Chip for the Real-Time Observation of Cellular Apoptosis. *Anal. Chem.* **87**, 12169-12176 (2015).
2. Lacy, P. & Stow, J.L. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood* **118**, 9-18 (2011).
3. Manz, R., Assenmacher, M., Pfluger, E., Miltenyi, S. & Radbruch, A. Analysis and sorting of live cells accordig to secreted molecules relocated to a cell-surface affinity matrix. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1921-1925 (1995).
4. Kihara, T., Ito, J. & Miyake, J. Measurement of Biomolecular Diffusion in Extracellular Matrix Condensed by Fibroblasts Using Fluorescence Correlation Spectroscopy. *Plos One* **8**(2013).
5. Masuelli, M.A. Hydrodynamic Properties of Gelatin –Studies from Intrinsic Viscosity Measurements. in *Products and properties of biopolymers* 85-116 (Verbeek C.J.R. , 2012).
6. https://en.wikipedia.org/wiki/Viscosity.
7. Goodhill, G.J. Diffusion in axon guidance. *Eur. J. Neurosci.* **9**, 1414-1421 (1997).
8. Li, B., Chen, J. & Long, M. Measuring binding kinetics of surface-bound molecules using the surface plasmon resonance technique. *Anal. Biochem.* **377**, 195-201 (2008).
9. Green, N.M., Anfinsin, C.B., Edsall, J.T. & Richards, F. *Adv. Protein Chem.*, (Academic Press, San Diego,CA, 1975).
10. Kusnezow, W., Syagailo, Y.V., Goychuk, I., Hoheisel, J.D. & Wild, D.G. Antibody microarrays: the crucial impact of mass transport on assay kinetics and sensitivity. *Expert Rev. Mol. Diagn.* **6**, 111-124 (2006).