Lab on a Chip

PAPER

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Cite this: DOI: 10.1039/c7lc00839b

Integrated platform for cell culture and dynamic quantification of cell secretion†

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We developed an automated microfluidic chip that can measure dynamic cytokine secretion and transcription factor activation from cells responding to time-varying stimuli. Our chip patterns antibodies, exposes cells to time-varying inputs, measures cell secretion dynamics, and quantifies secretion all in the same platform. Secretion dynamics are measured using micrometer-sized immunoassays patterned directly inside the chip. All processes are automated, so that no user input is needed for conducting a complete cycle of device preparation, cell experiments, and secretion quantification. Using this system, we simulated an immune response by exposing cells to stimuli indicative of chronic and increasing inflammation. Specifically, we quantified how macrophages respond to changing levels of the bacterial ligand LPS, in terms of transcription factor NF-κB activity and TNF cytokine secretion. The integration of assay preparation with experimental automation of our system simplifies protocols for measuring cell responses to dynamic and physiologically relevant conditions and enables simpler and more error free means of microfluidic cellular investigations.

Received 7th August 2017,
Accepted 27th October 2017

DOI: 10.1039/c7lc00839b

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Introduction

Cells continuously receive and respond to a wide variety of inputs. For instance, when immune cells are activated by pathogen sensing receptors, cytokines are produced and secreted to induce inflammation and activate the body's innate immune response.[1] Precise control of cytokine concentration is critical because abnormally high or low secretion of cytokines can result in inflammatory conditions, such as sepsis, or increased susceptibility to infection, respectively.[2] Prolonged inflammation can lead to other debilitating conditions, such as asthma, rheumatoid arthritis, multiple sclerosis, and cancer.[3] Tight regulation of cytokine secretion is thus essential for a healthy immune response.

The dynamics of cytokine secretion are important for tuning a cell's response in a larger population response.[3,4] For instance, it is predicted that neutrophils are recruited to the site of infection by propagating cytokine waves across tissue cells.[3] Moreover, pulsing a cytokine over a population of cells transforms unsynchronized transcription factor oscillations into entrained and synchronized oscillations.[4] Furthermore, an activated macrophage is able to transmit a wave of transcription factor activation across a population of fibroblast cells through cytokine secretion.[5] Undoubtedly, the dynamics of cytokines received and released from cells are important in understanding how populations of cells communicate with each other, and more specifically, how the immune system is coordinated. Nevertheless, due to technical challenges, the dynamics of cytokines released from cells given a dynamic input are not well understood. Even clonal populations of cells have heterogeneity in response to an immune stimulus.[6,7] This heterogeneity is an essential component in understanding how cells process biological noise to ultimately produce a robust response to infection.

Techniques exist to measure dynamic cytokine secretion from cells. However, these methods lack an all-in-one device to run dynamic cell experiments and immunoassays on the same platform. For instance, microengraving is a technique where cells are cultured in nanowells on a polydimethylsiloxane (PDMS) slab.[8–11] The PDMS slab is reversibly attached to an antibody-patterned glass slide used to measure secretion. Microengraving requires complex manual manipulation of (1) off-chip patterning of glass slides, and (2) detachment and replacement of a new glass slide for both changing media and measuring secretion for each time point. Furthermore, the final quantification of secretion using detection antibodies is completed separately off-chip. Single-cell barcode chips can also be used to measure dynamic cytokine secretion. In barcode chips, secretion is measured by seeding cells in nanowells on a PDMS slab, and successively replacing

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† Electronic supplementary information (ESI) available: Document with 4 figures and 1 movie. See DOI: 10.1039/c7lc00839b

antibody coated glass slides on the top of nanowells for each secretion time point. However, this device is not an all-in-one device, in that the DNA-encoded antibody libraries need to be patterned using an additional microfluidic device.[12–14] This limits the duration and feasibility of experiments and requires substantial manual involvement. We recently published a microfluidic chip that measures dynamic secretion from cells without the need to manually intervene to conduct immunoassays.[7]

In the current work, we improve on our prior design, by easing the chip set up to eliminate all off chip-steps necessary for experimentation. To use our previous chip, antibodies had to be attached to beads in additional off-chip processes. Subsequently, the beads had to be manually and sequentially loaded into the chip. By using an on-chip antibody patterning protocol, we cut down on many hours of manual labor needed to conduct experiments.[15–18] As a result, our new dynamic cell secretion chip saves researchers significant manual work and is more robust to errors as it does not require manual intervention in assay processes. The chip presented here is, to our knowledge, the first device that incorporates a full immunoassay, from antibody patterning to secretion quantification, of a cell culture exposed to dynamic stimuli. An all-in-one integrated and automated device is essential to simplify protocols, reduce operator time and errors, conduct long-term, complex, and multiday assays, and ultimately bring devices out of engineering labs and into the clinic and biology-focused labs.

Here, we present an integrated and automated platform that can expose cells to a dynamic input, track the cellular inflammatory transcription factor response, and measure the resulting cytokine secretion over time (Fig. 1). To test the platform, we studied how macrophages respond to infection. Specifically, we measured how RAW 264.7 macrophages respond to lipopolysaccharide (LPS), an inflammatory molecule found on the cell wall of Gram-negative bacteria. We measured the response by tracking NF-κB transcription factor activation, and TNF cytokine release. NF-κB is a major transcription factor in inflammation, which is known to control TNF secretion.[2] The platform improves on existing techniques by being able to precisely pattern micrometer-sized immunoassays, culture cells, and perform surface-based immunoassay quantification all in the same device. Furthermore, the device is adaptable in that immunoassay patterning, cell experiments, and immunoassay quantification are each automated, with no further manual manipulation needed after the reagents are connected to the device. The ease of operation of our current chip will facilitate further microfluidic exploration into how cells respond to dynamic inputs.

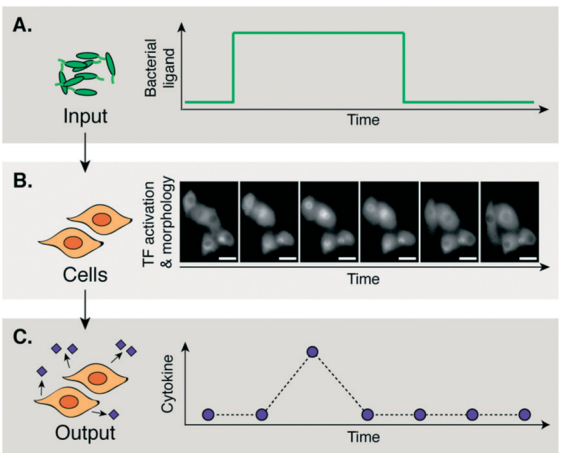

Fig. 1 Quantifying cell response to dynamic inputs. (A) The dynamic cell secretion chip can expose cells to a dynamic input. Cells are stimulated with a bacterial ligand to stimulate an infection. (B) Simultaneously, the transcription factor (TF) activation and morphology of the cells can be imaged every 5 minutes. Scale bar is 20 μm. (C) The cytokine secretion is measured every two hours.

Materials and methods

Chip control and operation

The device design and fabrication protocol was previously described in detail,[19] and further described in our ESI† materials and methods. The control channels of the dynamic secretion chip were selectively pressurized in order to control the movement of fluids in flow channels (Fig. 2, Fig. S1†). To operate the device, the control channels were filled with diH_2O and pressurized at 30 psi. The flow lines were operated at 5 psi, and valve switching was controlled with a custom MATLAB program. We developed a GUI with a corresponding library of scripts in order to implement automation in the device (available on request).[20] We extensively developed and built upon the simple open and close valve commands that comprised the basis of the control to fully automate chip assay functions.[20] An automated script works by timing the flow of various fluids through different parts of the chip.

Chip preparation

Preparation of a chip begins with purifying reagents, all of which were filtered through a 0.2 μm sterile filter before being added to the device. Air inside fluidic lines was then removed by debubbling the chip with PBS for 5 minutes. PBS was flowed into the chip, with a closed exit, until all bubbles were pushed out of the flow channels through the air-permeable PDMS. Then, cell chambers and button valves were closed, and 10 mg mL^{-1} pluronic (Pluronic F-127, Life Technologies) dissolved in water was flowed through the antibody spot chambers of the chip (Fig. 3Bi). This rendered all surfaces besides the cell chamber and antibody spotting regions non-adhesive. After pluronic treatment, the antibody spot chambers were extensively washed with PBS for 4 hours. Next, the cell chambers were coated with fibronectin to render the surface adhesive for cells.[19] 200 μg mL^{-1} fibronectin (FC010, Milipore, Zug, Switzerland) dissolved in PBS was flowed into the cell chambers, and incubated for the duration of the antibody patterning protocol (12 hours).

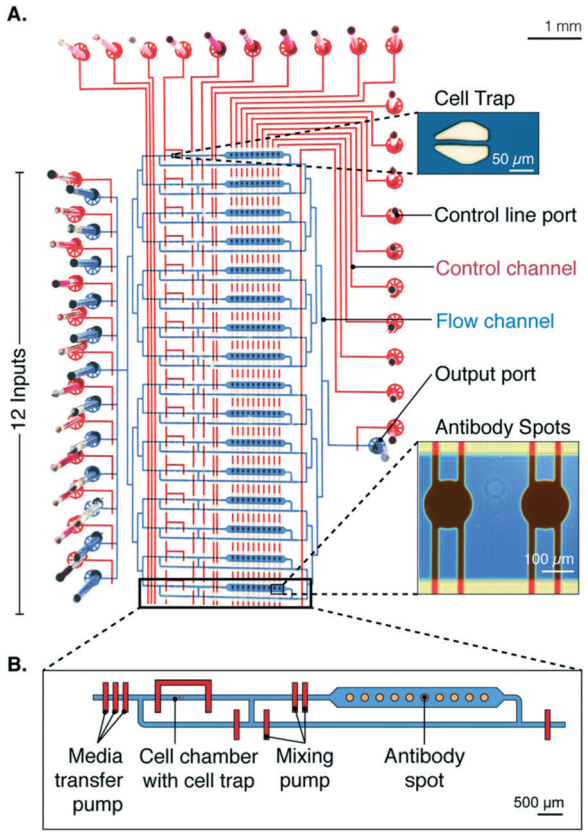

Fig. 2 Overview of dynamic cell secretion chip. (A) The chip has 16 units, 12 inputs, and 1 output. The control layer, marked in red, modulates the flow of fluids in the flow layer, marked in blue, where cells are cultured and the immunoassay is performed. (B) Each unit consists of a cell culture chamber, with a media transfer pump to feed cells, and an antibody spot chamber with 10 capture antibody spots used to measure the amount of cytokine in the cell culture supernatant. A separate mixing pump is used to mix the supernatant over the immunoassay (Movie S1†). Each cell culture chamber contains a cell trap.

Antibody spot patterning

Patterning of the antibody-based cytokine detecting spots was fully automated, and required no operator input after the reagents were connected to the device. The antibody patterning protocol was adopted from previous work.[15–18] In order to pattern an antibody, the antibody must only be biotinylated. To start the patterning protocol, the button valves were closed, and pluronic was flowed through the chip (Fig. 3Bi) as mentioned above. Next, the button valves were opened and 2 mg mL^{-1} biotinylated bovine serum albumin (BSA) (29130, Thermo Fisher Scientific) dissolved in distilled water (Fig. 3Bii), followed by 1 mg mL^{-1} neutravidin (3100, Thermo Fisher Scientific) dissolved in PBS (Fig. 3Biii), were flowed into the chip. The button valves were then closed, and biotinylated BSA was again flowed into the chip in order to bind to and passivate exposed neutravidin molecules surrounding the button valves (Fig. 3Biv). After this step, there is a neutravidin spot directly underneath the button valves. This specificity of patterning was confirmed by a fluorophore-tagged biotin molecule (Fig. 3Ci). Next, 7.5 μg mL^{-1} biotinylated anti-TNF (T9160-14, US Biological, USA) dissolved in PBS was added to the channels (Fig. 3Bv). Because of the neutravidin spot underneath the button valves, the antibodies were precisely patterned onto only to this spot, and the antibody spotting protocol was completed (Fig. 3Cii). The density consistency of capture antibodies was verified, and no significant difference in density was found between different buttons and rows (Fig. S2†). The integrity of remaining parts of the immunoassay, both the antigen and detection antibody addition, were also confirmed in Fig. 3Ciii and iv, respectively. Specific details of the patterning are described further in the ESI† materials and methods.

Addition of cells to device

After the antibody spots were patterned, the previously applied fibronectin was washed out of the cell chambers with fresh media. A cell suspension was then flowed through the chamber to seed cells before the start of the experiment. Cells were captured with a cell trap.[7] Cells were typically seeded at a concentration of 10^5 cells per mL, though cell populations as low as 5×10^4 cells per mL could also be successfully seeded, albeit this results in a lower density of cells per chamber. The cell seeding protocol is explained in detail in the ESI† materials and methods.

NF-κB measurement and analysis

We used RAW macrophages $p65^{-/-}$ with a p65-GFP reporter gene and H2B-dsRed nuclear marker to track cellular NF-κB activity.[2] In order to culture cells while maintaining simultaneous imaging capabilities, chips were operated on a Nikon Eclipse Ti inverted microscope inside of an enclosure with a fixed temperature of 37 °C with 95% humidity and 5% CO_2 (Life Imaging Services Basel, Switzerland). The cells were imaged using bright field and epifluorescence every 5 minutes during experiments. The NF-κB fluorescence of each cell was quantified by measuring the average fluorescent intensity in the cellular nucleus. Background fluorescence due to autofluoresence in the media was subtracted from cellular NF-κB measurements. Similar to our previous device, the NF-κB fluorescence of each cell was determined manually, and further downstream analysis was automated in MATLAB.[7] The background corrected measurements were normalized to the minimum and maximum fluorescence per cell during an experiment. Lastly, normalized measurements were smoothed using a moving average.

Secretion from cell

After connecting reagents to the chip, the secretion measurements were conducted with a fully automated script implemented in MATLAB. The script controls both the location and the timing of reagents in the chip. To quantify secretion, cellular supernatant was periodically moved from the cell chamber into the antibody spot chamber (Fig. 4A). First, PBS was flowed into the top part of the antibody chamber (Fig. 4A) for 5 minutes. Next, fresh media was flowed around

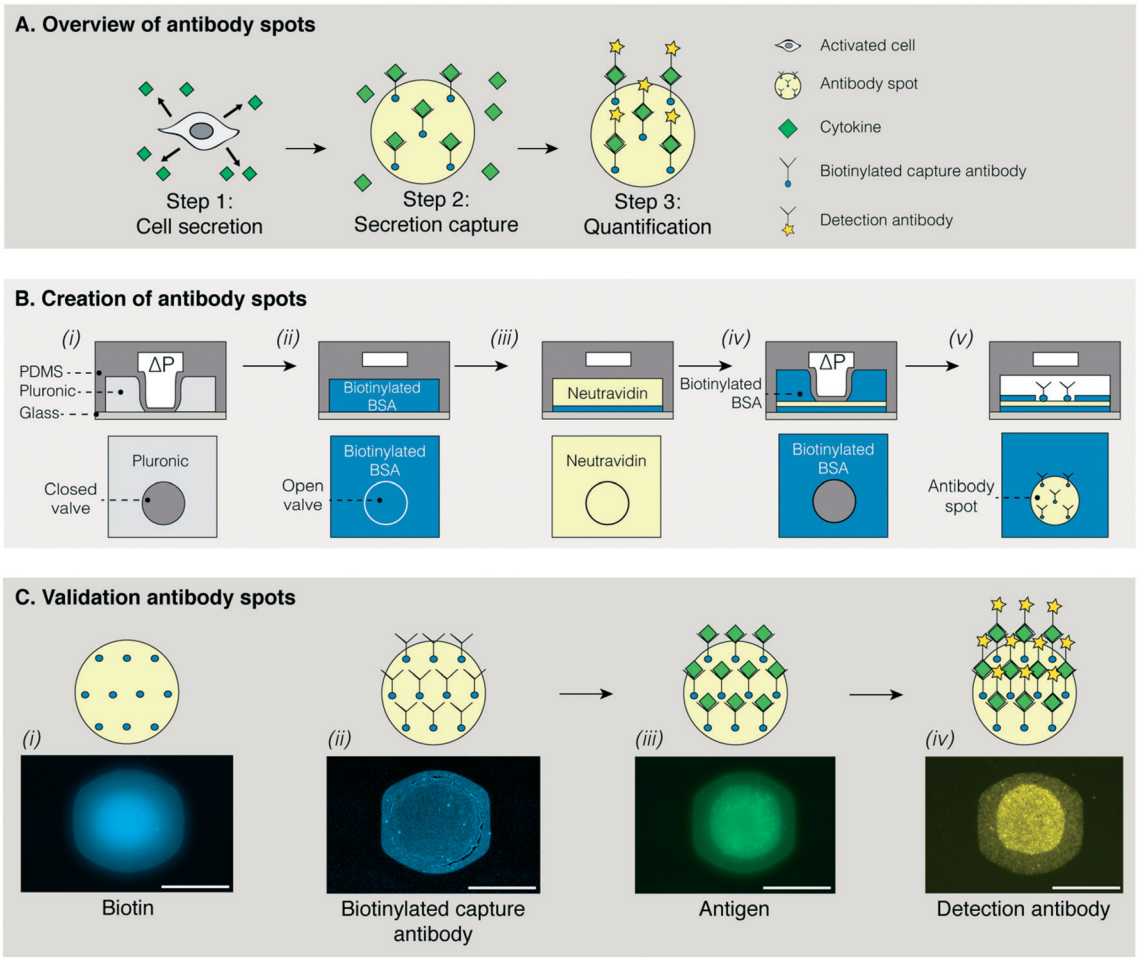

Fig. 3 On-chip patterned antibody spots for cytokine secretion quantification. (A) Overview of antibody spots. A cell secretes cytokines (step 1). Secreted cytokines are captured on antibody-coated spots (step 2). The number of cytokines bound to each spot is measured using a fluorescently labelled antibody (step 3). (B) Creation of antibody spots. The top images are representative of a cross section of the chip. The bottom images are from a birds-eye-view. The channel is initially passivated with pluronic (i) to create a non-adhesive surface outside the antibody spots. The antibody spots are then patterned by flowing biotinylated BSA (ii), and then neutravidin (iii) through antibody spot channels with open buttons valves. Next, the buttons valves are pressurized, and biotinylated BSA (iv) is flowed through the channel. This creates a neutravidin spot underneath the button valve. Lastly, the buttons valves are opened, and biotinylated anti-TNF antibodies (v) are flowed through the chip. The antibody spots are now patterned (v). (C) Validation of the antibody spots. The neutravidin spot patterned in (B.iv) is confirmed by flowing a fluorescent biotin solution through the chip. Fluorescent biotin binds robustly to the neutravidin spot. The existence of the initial biotinylated antibody spot was confirmed by using a fluorescent antibody against the antibody spot (ii), and using a labelled eGFP antigen (iii). A picture of a completed immunoassay on the spot is also shown (iv). Scale bar is 50 μm.

the cell chamber and the bottom part of the antibody chamber (Fig. 4Ai). This step was necessary to periodically replace the media in the cell chamber. When providing a cellular stimulus, a solution of LPS (L4524, Sigma-Aldrich Chemie GmbH, Switzerland) in media was instead flowed around the cell chamber. Next, the cellular supernatant was transferred to the antibody spot chamber. Specifically, the cell chamber was opened, and a peristaltic pump transferred the supernatant into the antibody spot chamber, and at the same time, transferred new media or media with stimulus into the cell chamber (Fig. 4Aii and S3†). Next, a button valve was opened, revealing an antibody spot, and the valves surrounding the antibody spot chamber were closed. One antibody spot was used per measurement time interval. A peristaltic pump was then used to mix the cell supernatant over the antibody spot (Movie S1†). The solution was mixed until adequate cytokine binding was achieved (Fig. 4D). After 90 minutes, the button valves were closed, and the antibody spot chamber was washed for 5 minutes with PBS. The process was repeated for each time point of cellular cytokine secretion.

Secretion calibration

Following cell experiments, a calibration with known amounts of TNF diluted into media was completed on chip (Fig. 4B and C). Specifically, the same protocol was used as for cellular measurements, except that solutions with known concentrations of TNF (PMC3014, Thermo Fisher Scientific, USA) dissolved in media were first flowed into the cell chamber. This process was repeated for each TNF concentration

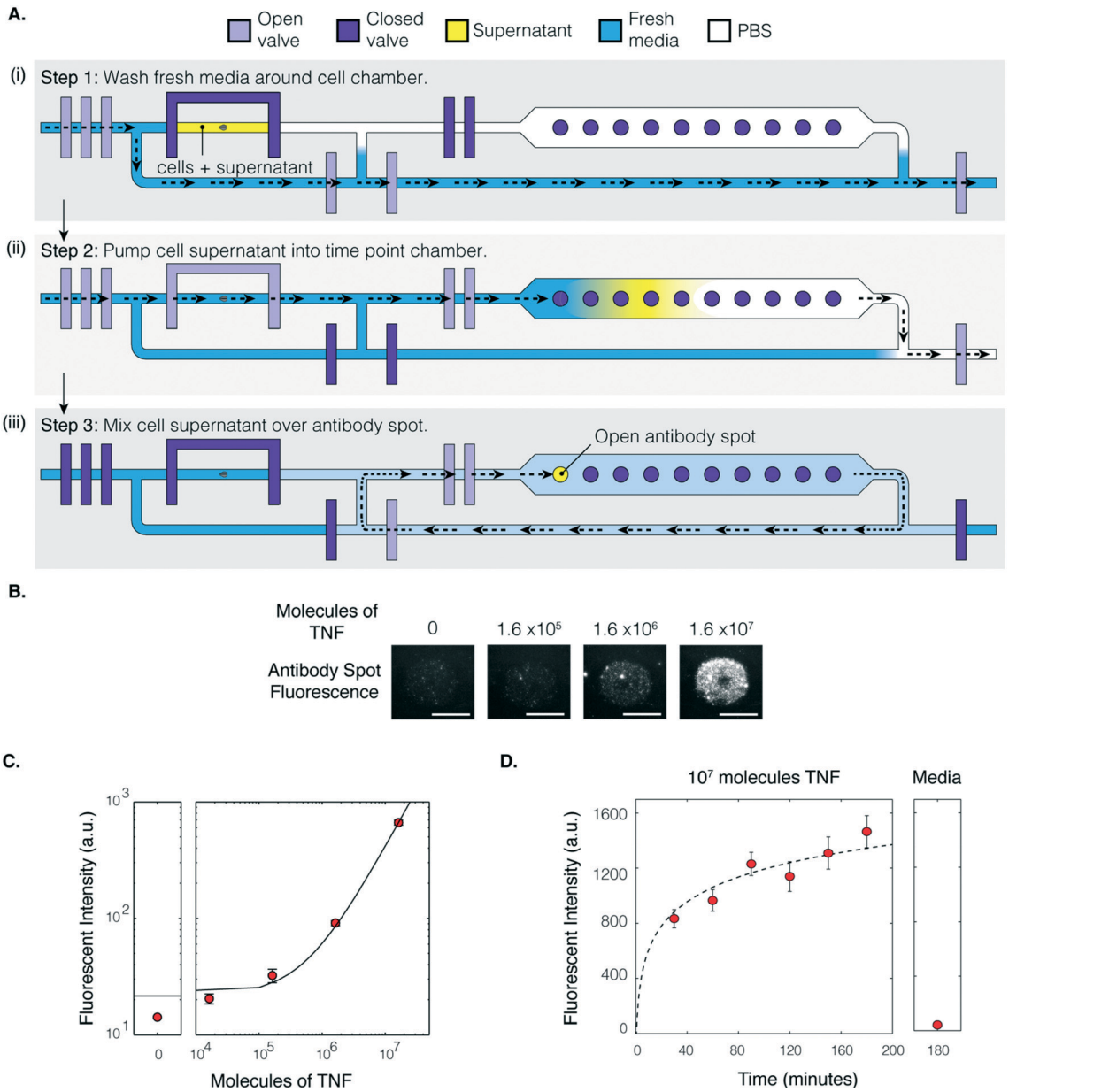

Fig. 4 The secretion profile is measured using an immunoassay. (A) (i) First, the cells are cultured in the cell chamber (yellow). New media (blue) is washed around the cell chamber. The antibody spot chamber is filled with PBS (white). (ii) After an incubation, new media (blue) is pumped into the cell chamber, and the cell supernatant (yellow) is pumped into the antibody spot chamber. The pumping parameters for successful transfer of supernatant from the cell chamber to the time point chamber are described further in Fig. S3.† (iii) In the antibody spot chamber, the cell supernatant is mixed over an exposed antibody spot, and the amount of TNF secreted by the cells is measured. A video of the mixing of a solution over an antibody spot is shown in Movie S1.† (B) Representative examples of antibody spots bound with varying amounts of TNF. Scale bar is 50 μm. (C) A calibration curve for TNF on the antibody spots. Error bars are the standard error of the mean. The limit of detection is ~155 000 molecules. (D) Dependence of antibody spot signal with mixing time. The time 10^7 molecules of TNF were circulated inside the chip was varied from 30 to 180 minutes. With increasing mixing time, the fluorescent intensity measured increased. As a control, media was mixed for 180 minutes over an open antibody spot. Error bars represent standard deviation. The dashed line is the line of best fit to a logarithmic curve.

tested. Using these calibration points, the amount of cytokine secreted by the cells was quantified. The limit of detection (LOD) is defined as the mean fluorescence intensity of media plus 3 times the standard deviation. The LOD of the dynamic secretion chip is ~155 000 molecules (10^{-19} moles) of soluble TNF. All calibrations were completed under identical conditions as cellular cultures; humidity, temperature, and surface coatings were all maintained.

Quantifying secretion

Two antibodies were used to quantify the amount of TNF captured on the antibody spots during measurements. The quantification process began by first washing out the antibody spot chambers with 0.05% PBS Tween (P7949, Sigma-Aldrich Chemie GmbH, Switzerland) for 20 minutes to remove unbound cytokines and other components from the

assay chamber. Next, 7.6 μg mL^{-1} of rabbit anti-TNF antibody (GWB-489500, GenWay Biotech, USA) in PBS was added with the button valves open. The anti-TNF antibody was flowed through antibody spot chambers for 5 minutes, and then incubated in the chambers for 10 minutes. This flow and incubation cycle was repeated twice, for a total of 30 minutes. The antibody chambers were then washed with 0.05% PBS Tween for 20 minutes.

Next, 3.3 μg mL^{-1} of fluorescent Cy3 labeled anti-rabbit IgG (I1903-12H, US Biological, USA) in PBS was flowed through the antibody spot chambers in order to visualize and quantify the amount of TNF bound to the spots. Specifically, the antibody was flowed through the channels for 5 minutes, followed by a 10 minute incubation. This process was repeated twice for a total of 30 minutes. Next, the antibody chambers were washed with 0.05% PBS Tween for 20 minutes, followed by PBS for 20 minutes. The antibody spots were then imaged using a Nikon Eclipse Ti inverted microscope. To quantify the fluorescence signal of each antibody spot, the mean fluorescence of each spot was measured using ImageJ. The spots were then normalized by subtracting the mean background signal next to each antibody spot from the measured mean fluorescence intensity of each spot.[21] The method for converting a measurement of fluorescence intensity to the number of TNF molecules is described further in the ESI† methods and Fig. S4.

Results and discussion

Device design

We designed an automated microfluidic chip that can expose cells to a dynamic input, quantify the cellular inflammatory transcription factor response, and finally measure the resulting cytokine secretion all in the same platform (Fig. 1). The microfluidic chip is composed of PDMS control and flow layers bonded to a glass slide in a push-down configuration (Fig. 2A). In this configuration, the cells were cultured and assays were performed directly on the glass surface. All protocols were automated with a MATLAB control software and corresponding GUI.[19,22]

The chip is capable of measuring the inflammatory transcription factor and cytokine secretion response of up to 16 groups of cells *via* parallelized units. Each unit consists of a nanoliter-sized cell chamber (920 pL), and an on-chip peristaltic media transfer pump to control the replacement of media in the cell chamber (Fig. 2B). Each cell chamber contains a cell trap in order to capture cells from a cell suspension for on-chip culture.[7] Furthermore, each unit contains 10 patterned antibody spots, which are used to measure cytokine secretion at 10 different time intervals, and an on-chip peristaltic mixing pump in order to increase the cytokine capture efficiency of the antibody spots (Fig. 2B).[7,23] In total, the device can measure 160 time points of cytokine secretion.

Cell secretion was measured with an immunoassay using on-chip patterned antibody spots (Fig. 3). Antibody spots were patterned with a capture antibody specific to a cytokine of interest. Later, cell supernatant was incubated over these spots, and secreted cytokines were captured on the spot (Fig. 3A). The amount of cytokine bound to each spot was quantified by a double sandwich immunoassay. All of these steps, from the initial patterning of antibodies, to the cell culture and secretion experiments, and to the final quantification of secretion using detection antibodies, were completed on-chip with fully automated control, meaning that no operator time or input was needed after the reagents were connected.

Capture antibody spots are patterned on-chip

Antibody spots were patterned on-chip using an adaptation of the mechanically induced trapping of molecular interaction (MITOMI) method.[15–18] This protocol uses button valves to selectively pattern antibodies directly underneath the valves. Unlike the traditional pneumatic microfluidic valves, which completely close a rounded flow channel, button valves are both narrower than the flow channel and are placed above a rectangular flow channel, and thus do not fully block the flow channel when closed (Fig. S1†). Button valves only block a small surface below the valve from exposure to external reagents, while allowing the rest of the flow channel to be exposed to a given solution (Fig. S1b†). Button valves are essential to the operation of the dynamic secretion chip by saving valuable functional chip area and allowing multiple antibody spots to be patterned adjacent to the cell chamber. Patterning in this way is additionally highly flexible in that the only requirement for placement of antibodies is that the antibodies are functionalized with biotin.

Two changes were made to the established MITOMI antibody patterning protocol.[15–18] First, instead of bonding the device to an epoxy coated glass slide, the PDMS chips were bonding directly to an uncoated glass slide. Patterning on epoxy slides, unlike glass slides, results in covalent attachment of antibodies.[15–18] Nevertheless, we patterned antibodies on glass slides in order to maintain good cell viability in our system. Second, the first reagent used for antibody patterning was pluronic (Pluronic F-127, Life Technologies), in order to make the flow channel surfaces, apart from the antibody spot regions, non-adhesive to proteins and other components within the medium. Pluronic was also necessary to make the antibody spot chamber non-stick for cell seeding into the cell chamber.

The antibody patterning protocol was run in an automated fashion. After the reagents were connected to the chip, a MATLAB script controlled the antibody patterning protocol and the protocol ran automatically for 12 hours. This is a significant improvement over our previously published method for dynamic cell secretion quantification,[7] which required up to 8 hours of manual loading of antibody beads. Moreover, this is also in contrast to the microengraving and barcoding methods, that require manual slide processing in order to coat antibodies onto glass slides.[8–10,12–14] The automation of antibody patterning described in our dynamic cell secretion

chip is a strong advantage of our technique due to manual time saved setting up the device for an experiment.

Cellular secretion profile measurement using antibody spots

The secretion profile of cells was measured using an on-chip sandwich immunoassay over the patterned antibody spots. Each antibody spot can be used for one time point measurement. To measure secretion dynamically, cell supernatant was periodically moved from the cell chamber to the antibody spot chamber (Fig. 4A), where one spot per time point is opened for the assay. By closing the button valve after each measurement, each antibody spot was mechanically blocked from further supernatant exposure. As a result, every antibody spot operated independently of one another, and multiple measurements could be performed in the same chamber by using a different antibody spot for each measurement.

We ensured no cytokine molecules were lost in this process by preventing diffusion losses by pumping cell supernatant directly into the antibody spot chamber (Fig. 4A). The cell chamber and antibody spot chambers are separated by a single valve, ensuring there was no loss of cytokines upon solution transfer. Moreover, the volume of the antibody spot chamber was ~23 times larger than the cell chamber. By pumping the supernatant into the middle of the antibody spot chamber, we further ensured that all of the supernatant reached the antibody spot chamber by continued pumping until new media behind the supernatant was also pumped into the antibody spot chamber (Fig. 4Aii and S3†).

Cells were exposed to dynamic inputs by changing the solution that flowed into the cell chamber (Fig. 4A) at each medium replenishment step. By having programmable and automated valves at input ports, it was possible to change the stimulus in the cell chamber within 5 seconds (Movie S2†). As a result, it is possible to use our device to expose cells to a wide range of possible input conditions, from short input pulses, to longer stimulations. By making use of all available input ports, it is possible to expose cells to up to 12 different stimuli.

Dynamic quantification of cellular NF-κB and TNF secretion response to LPS time courses

The dynamic secretion chip was used to expose cells to a dynamic input, track the cellular transcription factor response every 5 minutes, and track the resulting cytokine secretion

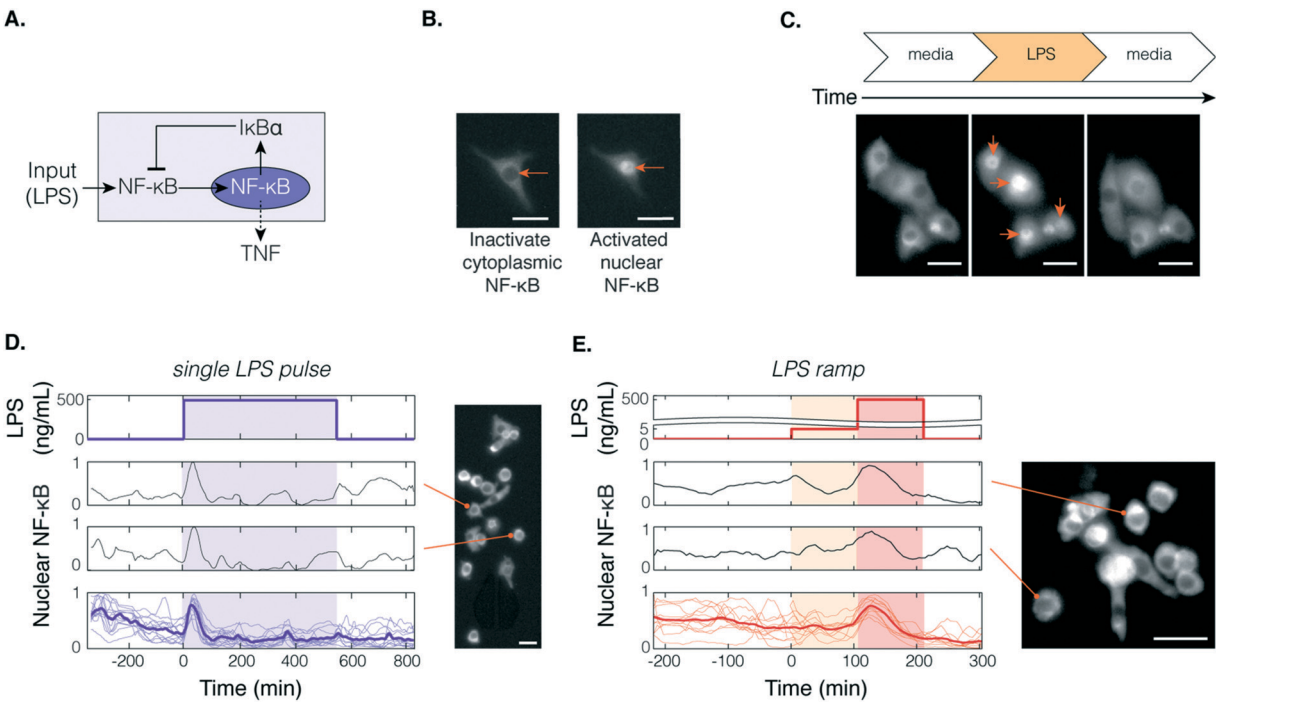

Fig. 5 The dynamic secretion chip tracking of the cellular NF-κB response to a variable input. (A) Exposure to LPS causes NF-κB activation and TNF secretion. When activated by LPS, NF-κB translocates from the cytoplasm (light purple) to the nucleus (dark purple). (B) Raw 264.7 macrophages with a p65-GFP reporter gene were used for experiments. During the cellular resting state, NF-κB is inactive and located in the cytoplasm. When NF-κB is activated, the transcription factor moves from the cytoplasm into the nucleus. (C) The dynamic secretion chip can be used to apply a dynamic stimulus to cells, and track the cellular NF-κB activation over time. When exposed to LPS, NF-κB translocates into the nucleus, as indicated with arrows. (D) The NF-κB response of 18 cells to a single pulse of LPS. Specifically, cells were exposed to 6 hours of media, 9 hours of LPS, and 3 hours of media. The media and LPS inputs are represented as a white and purple background, respectively. (E) The NF-κB response of 13 cells to an LPS ramp consisting of 4 hours media, 2 hours 5 ng mL^{-1} LPS, 2 hours 500 ng mL^{-1} LPS, and 3 hours media. The white, light orange and dark orange backgrounds indicates when media, 5 ng mL^{-1} LPS, and 500 ng mL^{-1} LPS, respectively, were in the cell chamber. In panels (D) and (E), the stimulus input is shown in the top graph, the trace of two cells from the population is shown in the middle two graphs, and the average cellular response shown by the bolded line in the bottom graph. The thin lines in the bottom graph are the NF-κB responses of all cells in the chamber. Scale bar is 20 μm.

every 2–3 hours. It is meaningful to observe the secretion patterns of small populations of cells, as paracrine signaling is important to recapitulate a strong response to LPS in macrophages.[14,24] Specifically, we studied how cells process a LPS input through the NF-κB transcription factor, and finally to a TNF cytokine output (Fig. 5A). Activation of NF-κB leads to the secretion of proinflammatory cytokines. For instance, NF-κB is known to control TNF secretion,[2] and TNF is one of the first proinflamatory cytokines secreted by macrophages in response to a pathogen.[25]

The cellular NF-κB response was tracked by using RAW 264.7 macrophages labeled with a p65 GFP marker.[2] An example time course of a media stimulus, followed by a 500 ng mL^{-1} LPS, and then a media stimulus is shown in Fig. 5C. Note how during the media stimulations, NF-κB is mainly found in the cytoplasm, and how the NF-κB translocated to the nucleus during the LPS stimulation. The first NF-κB and secretion measurements were always done under baseline conditions, with fresh media. This was necessary to understand the initial cellular state before stimulus addition. Examples of two stimulus patterns given to cells are shown in Fig. 5D and E. First, we exposed cells to a time course that mimics a strong, chronic inflammatory state (Fig. 5D). We exposed cells to media, 9 hours of high concentration LPS, and lastly media. We were able to track NF-κB dynamics on a single cell level with 5 minute resolution over 20 hours. Additionally, we exposed cells to increasing inflammation, by exposing cells to media, a low concentration of LPS, a high concentration of LPS, and finally media (Fig. 5E). In the increasing stimulus condition, macrophages only had a NF-κB peak response to the high concentration of LPS.

We observed that the cellular NF-κB response to a strong and chronic inflammation was similar to the cellular response to an increasing ramp of LPS exposure. In both conditions, no difference was observed in the peak response to the high 500 ng mL^{-1} LPS stimulus. The single cell response to a strong and chronic stimulus resulted in a NF-κB peak 22 ± 1.4 minutes after stimulation, and similarly the increasing inflammation condition had a peak 20.5 ± 1.6 minutes after stimulation. The peak timing of NF-κB in response to LPS

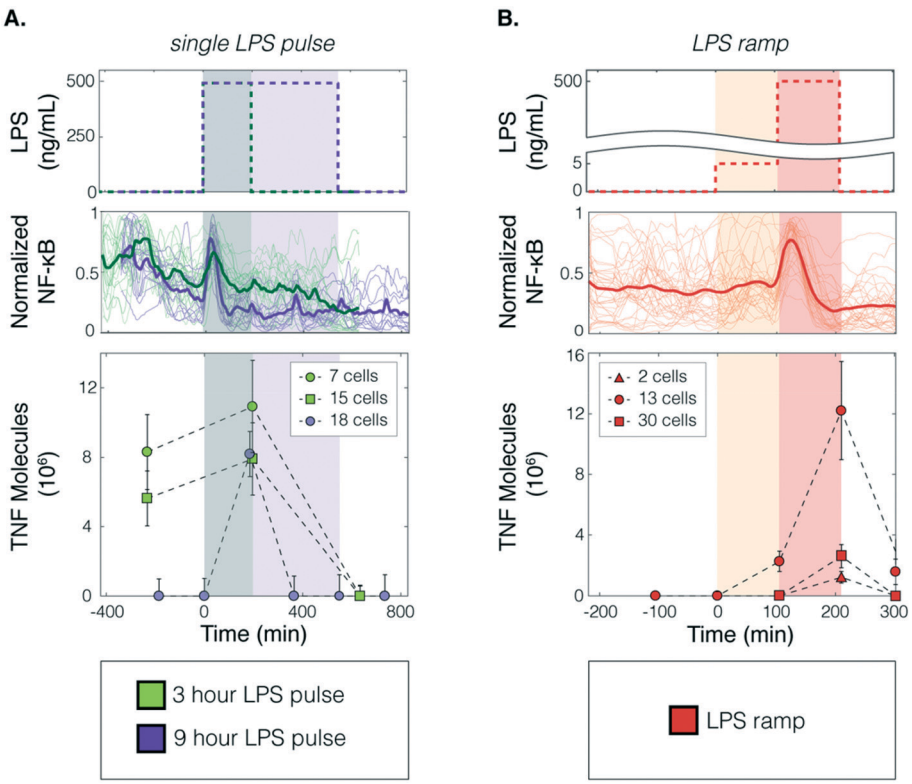

Fig. 6 Coupled cytokine secretion and transcription factor response of cells to a dynamic LPS input. Cells were simulated with an LPS time course, indicated by the top graphs. The middle panel shows the NF-κB response of all cells. The thin lines represent the single cell traces. The bold lines are the average cellular response. The cellular TNF secretion response is shown in the bottom graph of both panels. Time zero indicates the start of the LPS pulse. The top, middle, and bottom graphs all have the same time course, as indicated by the time course in the bottom graph. (A) 40 cells were stimulated with a single pulse of 500 ng mL^{-1} of LPS. Cells were exposed to an LPS pulse of either 3 hours (green) or 9 hours (purple). Cells were exposed to media preceding and following the LPS pulse. The media, 3 hour LPS, and 9 hour LPS inputs are indicated by a white, green, and purple background, respectively. The TNF secretion of cells from 3 experiments is shown in the bottom panel. (B) 45 cells were stimulated with two doses of LPS (LPS ramp). Specifically, cells were stimulated with (1) media, (2) a dose of 5 ng mL^{-1} LPS, (3) a dose of 500 ng mL^{-1} LPS, and (4) media. The media, 5 ng mL^{-1} LPS and 500 ng mL^{-1} LPS inputs were represented by a white, light orange and dark orange background, respectively. The TNF secretion of cells from 3 experiments is shown. In the bottom graph of both panels, the error bars represent the standard error of the maximum likelihood that a certain fluorescence value equals a value of TNF secretion. This is described further in Fig. S4.†

closely matched published results, where NF-κB activation was observed 24 minutes after stimulation of RAW 264.7 macrophages with LPS.[2]

Variability in macrophage activity was also studied through the standard deviation of the cellular NF-κB response (Fig. S5†). Variability in macrophage NF-κB activity decreased after exposure to the high dose of LPS in both the chronic and increasing inflammation conditions. Variation in NF-κB activity was highest during the first media stimulus (Fig. 5D and E and S5†). This is likely due to stress on the cells after seeding. For this reason, stimulation experiments with LPS always started at least 3 hours after cell seeding.

On the same set of cells, we were also able to measure TNF secretion (Fig. 6). Both stimulation conditions resulted in a single peak of TNF secretion following LPS exposure (Fig. 6). A single high peak in response to LPS stimulation matches previous results,[7] and we hypothesize that we only observed one TNF peak because upon cellular activation, the cellular TNF mRNA stockpile is rapidly translated, transported to the cellular membrane, enzymatically cut and rapidly secreted from cells.[26] After the peak in TNF secretion, cellular NF-κB relocated from the nucleus back to the cytoplasm. Deactivation of cellular NF-κB has been suggested to decrease levels of proinflamatory cytokine expression, such as TNF.[2] In one experiment of the increasing inflammation condition, we observed a gradual release of TNF (Fig. 6B). This may be a result of the lack of cellular NF-κB activation during the low concentration of LPS exposure, and subsequent peak of activation after stimulation with the high concentration of LPS. We thus observed that only after NF-κB peak activation, is there a maximum peak in TNF secretion.

These results show that we are able to use the dynamic secretion chip to track cellular response to dynamic stimuli *via* coupled NF-κB activation and TNF secretion. We were able run cell experiments in a fully automated fashion over 20 hours with a resolution of 5 minutes for NF-κB signaling and 2–3 hours for cytokine secretion measurements.

Conclusions

We developed an integrated device that is able to expose cells to dynamic inputs, study the cellular transcription factor activity, and track the resulting cytokine secretion over time. To our knowledge, this is the first platform that has full automation of immunoassay preparation, cell secretion experiments, and secretion quantification all in the same device. Unlike microengraving, barcode-based chips, and our previously published chip, here, total immunoassay preparation, experimentation, and readouts are conducted automatically on chip, without the need for complex manual manipulations.[7–10,12–14] We used this integrated platform to measure how cells respond to two different stimuli, an extended and high concentration of LPS, representing a chronic infection, and an increasing ramp of LPS, representing increasing inflammation. This showed, quantitatively, how cells sensed and differentiated dynamic inputs. Moreover, our device has the potential to work with rare cell populations, such as circulating tumor cells and rare stem cells as even low concentrations of cells can be seeded into the device.

Our device will allow further deciphering of how cells control cytokine secretion in response to dynamic stimuli. Precise control of cytokine secretion is essential in order to further study how cells communicate with each other to coordinate a robust response to infection. This is especially important considering that the latest breakthroughs in immunological therapies, such as engineered T cell therapy, rely on understanding how immune cells compose a potent attack specifically against cancer cells.[27] In response to such therapy, many patients have experienced a cytokine release syndrome side effect, characterized by uncontrolled cytokine secretion, that has resulted in organ failure, cardiotoxicity, and, in some cases, death.[27] Understanding the cytokine response of engineered immune cells is thus critical for further developing such new technologies. The integration and automation provided by our device makes it well suited for studying cellular responses to dynamic stimuli in the clinic or in traditional biology labs.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We would like to thank Erica Montani and Thomas Horn from the ETH D-BSSE Single Cell Facility for help with the microscopy involved in the project. Further, we would like to thank Felix Franke for help with the statistical analysis, and Tino Frank, Sebastian Bürgel, and Kasper Renggli for a critical review of the manuscript. An ERC Starting Grant (SingleCellDynamics) to ST funded this research.

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