A microfluidic live cell assay to study anthrax toxin induced cell lethality assisted by conditioned medium

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It is technically challenging to investigate the function of secreted protein in real time by supply of conditioned medium that contains secreted protein of interest. The internalization of anthrax toxin is facilitated by a secreted protein Dickkopf-1 (DKK1) and its receptor, and eventually leads to cell lethality. To monitor the dynamic interplay between these components in live cells, we use an integrated microfluidic device to perform the cell viability assays with real-time controlled culture microenvironment in parallel. Conditioned medium, which contains the secreted proteins from specific cell lines, can be continuously pumped towards the cells that exposed to toxin. The exogenous DKK1 secreted from distant cells is able to rescue the sensitivity to toxin for those DKK1-knocked-down cells. This high-throughput assay allows us to precisely quantify the dynamic interaction between key components that cause cell death, and provide independent evidence of the function of DKK1 in the complex process of anthrax toxin internalization.

Anthrax, a lethal infectious disease for human and other animals, is caused by anthrax toxin that is secreted from Bacillus anthracis1,2. Anthrax toxin consists of three proteins; protective antigen (PA) and two toxic factors: lethal factor (LF) and edema factor (EF)3,4. None of these three proteins is toxic when it exists alone. However, PA can form toxic complex with LF or EF, and subsequently enters the target cell cytoplasm through anthrax toxin receptor-mediated endocytosis and finally causes the cell death5–7.

It is still challenging to identify additional key components that are related to the internalization and lethality of anthrax toxin. Further investigation of the internalization mechanism of anthrax toxin will not only decipher the missing components in the pathological processes, but facilitates the discovery of therapeutic targets. It has been reported that low-density lipoprotein receptor-related protein 6 (LRP6) can mediate the internalization and lethality of anthrax toxin8. LRP6 is also a receptor for a secreted protein Dickkopf-1 (DKK1)9–11. In Wnt signaling pathway, DKK1 can form ternary complex with LRP6 and Kremen2, another membrane protein, for internalization12,13. We have recently reported that DKK1 plays an important role in the internalization and lethality of anthrax toxin through the ternary structure of LRP6-DKK1-Kremen214. However, it is still technically challenging to investigate the function of secreted protein in real time without toilsome protein production and purification processes. One alternative approach is to use conditioned medium that contains secreted protein of interest. However, it is technically difficult to perform reproducible experiments with cells cultivated with conditioned medium, which is typically collected from other cell culture15,16. Moreover, medium acquisition and storage are often time-consuming and labor-intensive, making large-scale or multiplex experiments difficult to perform.

Microfluidics becomes an ideal technology to reduce the reaction volumes for highly parallel assays17–21. Recent advances in microfluidics have greatly improved the controllability and precision, enabling the dynamic and programmable microenvironment control of cell culture, especially for small number of cells22–25. Microfluidic devices have been applied to study the cell responses to secreted factors in the culture medium through two major ways: 1) create a chemical gradient, stable or dynamic, of soluble molecules by micro-patterns or well-regulated flows on-chip26–28, 2) constrain the physical distribution of different types of cells and control the direct or indirect interactions between cells29–33. Neither of these approaches is cost-effective to supply fresh conditioned medium, nor can they easily avoid the direct attachment between different types of cells.
Here we report a novel approach to accurately investigate how cells respond to the conditioned medium with specific soluble factors secreted from other live cells. We employed this approach to study the role of DKK1 in cell susceptibility to anthrax toxin. We identified a toxin-resistant LM/shRNA157B cell line, whose DKK1 expression level has been suppressed, and dynamically measured its viability under different culture conditions, with and without the exogenous DKK1 supplies (Figure 1a). These DKK1 proteins, freshly secreted from the wild-type LM cells that were cultured on-chip in a remote chamber, could be actively pumped into the culture chambers with DKK1-knocked-down cells. We found that exogenous DKK1 effectively rescued the cells’ lost sensitivity to toxin, suggesting that DKK1 was likely serving as an activator in the internalization of anthrax toxin and consequently the cell death. Our high-throughput device could perform 4 groups of experiments, all with replicates, in 128 cell-culture chambers and 96 medium replacement chambers, providing an advanced technical solution to study the interaction between cells and soluble factors in the medium.

**Results and discussion**

We fabricated the device using PDMS majorly because of its two intrinsic advantages: 1) PDMS is gas permeable, allowing air and CO₂ to reach the medium in the closed culture chambers; 2) PDMS is optically transparent with negligible background fluorescence, making the microscopic observation convenient and quantitative. To better support the device, the chip was placed on a glass slide. To quantitatively study the biological function of proteins secreted from live cells, we carefully considered the layout and dynamic interactions between culture chambers that embedded in the device. Cells cultured in one chamber would not physically attach to the cells in other chambers while the pre-designed fluidic channels, with pneumatic valves to define the open/close status, linked the chambers.

The layout of the whole device is shown in Figure 1b. The fluidic layer of the chip is illustrated as color-filled patterns. The whole chip has 4 major inputs (C1 to C4) to load cells into the culture chambers. Each input is responsible for 32 cell-culture chambers. There are also 12 inputs on the chip to introduce the culture medium. The control layer of the chip consists 22 control lines that can actuate 423 integrated pneumatic valves. Among them, 144 valves form 48 peristaltic pumps, which are divided into 6 groups.

The chip can be viewed as the integration of 4 identical sections, and each section has 24 chambers for medium replacement and 32 chambers for cell culture, forming 4 identical replicating functioning units, one of which is highlighted in Figure 1b. The detailed structure of a functioning unit is illustrated in Figure 2a.

Every chip has 128 rectangular cell culture chambers. Each chamber is 1.20 mm × 1.30 mm × 0.05 mm, suitable to contain ca. 1,000 cells. The medium replacement chamber is 1.70 mm × 1.30 mm × 0.05 mm. 2 medium replacement chambers supply 4 cell culture chambers. Figure 2b show the key function of this device: fluidic...
exchange control between chambers. We load the top two chambers (1 and 2) with one type of cells and bottom two (5 and 6) with another type, leave the central two (3 and 4) free of cells. In this 2 × 3 configuration, every chamber is connected with the adjacent chamber with a short microfluidic channel, 300 μm wide and 25 μm high. These channels, with integrated pneumatic valves to open or close, are designed for medium exchange between chambers. One three-valve peristaltic pump has also been placed in this 6-chamber structure to drive the fluidic flow. We have found that medium refreshment is critical to keep cells healthy, and the refreshment may need to be more frequent than conventional culture due to the very limited volume in each culture chamber. The chamber 3 and 4 were used to introduce fresh medium and replace the old one (Figure 2c). Then the replaced medium was mixed with the old one in the other 4 chambers by actuating the peristaltic pump (Figure 2d), and distributed uniformly in a few minutes among all the connected chambers (Figure 2e). In this 6-chamber configuration each operation will replace part of the medium, which brings sufficient nutrient, removes considerable amount of metabolic wastes, and still keep a big portion of the secreted soluble factors in the medium. Each functioning unit has a 6-chamber structure to mix the media from two types of cells, and two 4-chamber structures with similar designs serving as the control groups for both cell types (Figure 2a).

The cell culture chambers were not just plain square boxes. We placed two bars in each chamber, shown in Figure 2f, and turned the chamber into a 3-section zigzag channel. This design was proved to be a simple yet effective solution which ensured that there would not be any stationary part in the chamber when medium flowed through, generating evenly seeded cells and uniform medium exchange in each chamber. The flow characteristics of square and zigzag cell culture chambers, and the cell seeding results were shown in Figure 2f. White arrows indicated the stationary spots that cells rarely seed onto. The DKK1-knocked-down LM/shRNA157B cells and their corresponding control groups (wild type LM cells) were employed in our experiment. Every functioning unit contains 8 cell culture chambers, 4 loaded with wild type LM cells and the other 4 with LM/shRNA157B cells. We hence had 4 groups of experiments in parallel: LM cells (Group A), LM cells that shared secreted DKK1 with LM/shRNA157B (Group B), whose DKK1 expression level has been suppressed (Figure 3a), LM/
shRNA157B cells with exogenous DKK1 freshly secreted from LM cells (Group C), and LM/shRNA157B cells without exogenous DKK1 (Group D). Each group has two technical replicates, and in total we have 4 biological replicates in each section. 12 inputs, 3 in each section, were applied to introduce the culture medium. Within one section we kept the medium identical while between sections, the concentrations of anthrax toxin were different.

We tested the performance of medium mixing that driven by peristaltic pumps embedded on-chip. Culture media with different toxin concentrations were loaded into the pre-designed sections, respectively. A typical mixing took 10 min, through a 50 Hz pumping action using the integrated 3-valve peristaltic pumps. After mixing the medium was uniformly distributed among all the connected chambers in both the experimental and control groups.

In each device, we used 4 sections to simultaneously carry out the experiments with 3 toxin dosages, and an extra group without toxin as the reference. Cells that died during the experiment would detach from the chamber bottom and were then flushed out of the device with other metabolic wastes through medium mixing and changing. The LM/shRNA157B cells in Group C would not produce sufficient DKK1 while they could continuously receive extracellular DKK1 secreted from the cells in Group B. In conventional practice, we replace the toxin-containing medium with fresh medium after 48 h treatment. However, when cultured on chip, the chamber is only 0.05 mm thick so we have to introduce the fresh medium more frequently to keep the cells healthy. We noticed that medium replacement every 3 to 6 h is necessary to maintain the cell viability at expected cell density in culture chamber. To minimize the DKK1 concentration fluctuation, we chose to replace the medium every 6 h.

We examined the cell viability on-chip through the live/dead staining with Calcein AM (a green fluorescent dye for indicating live cells) and Ethidium Homodimer-1 (EthD-1, a red fluorescent dye for indicating dead cells via nucleic acid labeling). In every section, we kept the medium identical while between sections, the concentrations of anthrax toxin were different.

We then monitored the dynamic response of the cells to toxin to fully exploit the advantages of large-scale integrated microfluidic devices. The chip was incubated in the homemade culture chamber on the sample stage of a microscope. The chamber could maintain an environment of the mixture of air and 5% CO2 at 37°C without interfering microscopic observation and image acquisition at the same time. We initiated the toxin assay when the cells grew to confluence on-chip. We took time-lapse bright-field microscopic images of each chamber when toxin-containing medium was introduced into the chip, and at the time points of 12 h, 24 h, 32 h, and 40 h.

Finally, we stained all the chambers with Calcein AM/EthD-1 at 48 h.
When we used 25 ng/ml of PA and 50 ng/ml of FP59, wild-type LM cells (group A) exhibited a clear dead trend at 24 h after toxin was provided, while at that time all the other three groups did not show obvious cell death. Later (32 h), we found that the cells in group B began to shrink and the shape turned to round. Similar morphological changes had also been observed in LM/shRNA157B cells of in group C, but with a time delay of 8 h. This time difference was probably due to the difference in DKK1 concentration. Through the real-time observation of the cell status in microfluidic devices, we could obtain the accurate information of cells’ dynamic response to toxin, and provide extra evidence in analysis of the biological functions of DKK1 in anthrax lethality.

**Conclusions**

In summary, we have presented a novel microfluidic live-cell assay to quantitatively study cell response to toxin using continuously supplied conditioned medium that produced by a different group of cells in real-time. All the cells were cultured on-chip and the secreted factors could be specifically delivered into certain cells through microfluidic fluid manipulation. We applied this assay to study the effect of a secreted protein, DKK1, on cell susceptibility to anthrax toxin in LM/shRNA157B cells, in which the endogenous DKK1 expression had been knocked-down. Highly integrated micro-valves and micro-pumps made the multiplex experiments possible. We have realized 128 parallel culture chambers, as well as 96 medium replacement chambers, to operate simultaneously, with two types of cells, four toxin concentrations, and four replicates. This compact device was fully automated by computer programming with real-time microscopic observation in-situ. Our demonstration has shown that experiments that are conventionally difficult to operate or hard to reproduce, such as the cell culture requiring precisely and dynamically controlled conditioned medium, can be readily performed.

**Figure 4** | The cytotoxicity assay of microfluidic-cultured cells with DKK1-expressed conditioned medium. (a) Images of cells at the end of toxin assay, with different PA concentration. For each culture chamber, phase contrast image and fluorescence images for Calcein AM and EthD-1 are captured for analysis. (b) Survival rates of cells that treated with PA at different concentrations. (c) Time-lapse microscopic images (phase contrast, and fluorescence for both Calcein AM and EthD-1) at the time points of 12, 24, 32, and 40 h for each chamber. Red boxes highlight the morphological changes associate with the beginning of cell death (50 ng/ml of FP59 is supplied with PA in each assay). Scale bar: 20 µm.
Applied this platform, with much lower consumption of materials and much higher accuracy and reproducibility.

**Methods**

**Conventional cell culture.** All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen) and 50 units/mL of penicillin-streptomycin (Invitrogen), and incubated in a humidified incubator containing 5% CO2 at 37 °C.

**Cell transfection.** Lentiviral shRNA expression constructs plKO.1 carrying shRNA specifically targeting on mouse DKK1 gene were obtained from OpenBiosystems, Inc. DNA transfections were performed with Polyethyleneimine (PEI)-mediated method. Lentiviruses were produced by transient transfection of HEK293T cells by shRNA expression construct with pCMV.R8.74 and pMD2.G-VSVG. Stable shRNA expression LM cells were achieved through viral infection followed by puromycin (2 μg/mL) selection. Real-time PCR was performed to confirm that mDkk1 expression was decreased in LM cells.

**Off-chip cytotoxicity assay.** PA were produced using plasmid pET-22b-PA and FS9, a surrogate of LF consisting of the N-terminal 1–254 residues of FS9 fused to the catalytic domain of Pseudomonas aeruginosa exotoxin A, was produced from List Biological Laboratories, Inc. Cytotoxicity assays were performed as described with MTT (AMRESCO). Each data point and related error bar shown in the figure for the MTT assay represent the average results from six replicates.

**Real-time PCR.** RNA of cultured cells was isolated using EasyPure RNA kit (Transgen, BE101-01), and the cDNAs were synthesized by PrimerScript 1st Strand cDNA Synthesis kit (TAKARA, 610IA). Real-time PCR was performed with Brilliant III Ultra Fast SYBR Green QPCR Master Mix (Stratagene, 608088) on Stratagene MX3005P qPCR system. β-actin transcript levels were measured as internal controls.

**Sequences.** Mouse DKK1 target of lentiviral shRNA: AGAGCCATCATGTGGAGCCGTCTTTTG. PCR primers for mDkk1: 5′-ATGAGGGCCAGGAAGTGAC-3′, 5′-GACCTCTGCTCTTGTTTGT-3′. PCR primers for mouse β-actin: 5′-CCAGCCCTCTCTGTTGAT-5′, 5′-GTAACGTCCGCGTGAAG-3′.

**Fabrication of multilayer microfluidic devices.** All devices were fabricated using multilayer soft lithography. Devices are composed of three layers of polydimethylsiloxane (PDMS, RTV615 kit, GE), bonded to a cleaned glass slide (7 × 10 cm). Two separate master molds, one for the fluidic layer and the other for the control layer, were fabricated by photolithography. The silicon wafers were treated with hexamethyldisilazane (HMDS, Alfa Aesar, USA) vapor for 3 min at 25 °C. The hybrid master mold of the fluidic layer was fabricated through a multi-step photolithography to form the molds with different patterns. The flow channels were fabricated by double spin-coating positive photoresist (PA620, AZ Electronic Materials) to a thickness of 25 μm. After photolithography, the patterned positive photoresist was re-flowed on a hot plate ramped from 30 °C to 220 °C at 6 °C/h, to obtain rounded channel profiles, with a peak height of about 25 μm. Subsequently, the chambers for connection were patterned by 15 μm thick negative photoresist (SU-8 2025 MicroChem, Newton, MA). Finally, the hybrid master mold was baked at 160 °C for 1 h to fully crosslink the SU-8. The thickness of the SU-8 layer had 15 μm of height, corresponding to positive photoresist (AZ Electronic Materials). Before the fabrication of PDMS chips, both molds were treated with trimethylchlorosilane (TMCS, Sinopharm, China) vapor for 3 min at 25 °C. The fluidic layer was made by pouring PDMS (5:1, elastomer to crosslinker ratio) onto its mold to a thickness of 5 to 6 mm. The control layer of the chip was made by spin-coating PDMS (20:1, elastomer to crosslinker ratio) onto the mold at 1400 rpm for 60 s. Then the fluid and control layers were baked at 80 °C for 20 min and 30 min, respectively. After the fluid layer was peeled off from its mold and hole-punched, it was aligned over the control layer, and then bonded at 80 °C for 45 min. The bonded layers were peeled off from the control mold, hole-punched, then placed on a glass slide with a thin, cured PDMS layer (10:1, elastomer to crosslinker ratio). Finally, the whole chip was incubated at 80 °C for at least 6 h. The complete chip is shown in Figure S1.

**Automation.** All the valves in the chip were driven by computer controlled solenoid valves. When the process of medium exchange was confirmed, the procedure was performed automatically using a designed LabVIEW (National Instruments, Austin, TX, USA) program. All imaging instruments were controlled by our own program script written in MATLAB (MathWorks, Natick, MA, USA).

**Microfluidic cell culture.** At the beginning of each experiment, Pluronic F-127 (Sigma-Aldrich; 0.2% w/w in PBS, filter-sterilized) was incubated for 1 h inside the device. Cell viability is determined by staining with two-probe solution which contains 2 mM Calcein AM (Invitrogen) in PBS. The stain solution is incubated for 30 min before imaging.

**Image taken and analysis.** Phase-contrast and fluorescence images of cells viability in each chamber were recorded by an automated microscope (TE2000E, Nikon) with a CCD camera (Olympus, DP72). Time-lapse images of cell growth and death curve were obtained at the time point 0, 12 h, 24 h, 32 h, 40 h and 48 h after toxin added. Home-developed MATLAB scripts were employed to perform image analysis and data statistical analysis. We developed our own MATLAB script to recognize and calculate the cell survival rate from fluorescent images. We picked up the red channel from the images labeled with EthD-1, and then summed total red pixels, representing dead cell numbers. The total green pixels were calculated according to the same method, representing live cell numbers. Since the enzyme was not completely inactivated, some dead cells were marked with green and red at the same time, so we defined the double labeled cells as dead cells, that is, cytotoxicity assay of the living cells is green pixels minus double labeled pixels. Because lots of dead cells were off the base, and washed away when the culture medium updated, we considered the live cells number in the control group as a starting cell numbers in experimental group. The survival rate was calculated by dividing the number of the living cells in experimental group by that in control group.

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Author contributions
Y.H. and W.W. conceived and designed the methods and experiments. J.S., C.C., Z.Y., Y.P., Y.Z. and L.Q. conducted the experiments and data analysis. J.S., W.W. and Y.H. wrote the manuscript.

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