MEASUREMENT OF INFLAMMATORY CYTOKINE SECRETION FROM HUMAN MONOCYTES AFTER INFLAMMASOME ACTIVATION

Yoshitaka Shirasaki[1, 2], Asahi Nakahara[2], Nanako Shimura[1], Kazushi Izawa[3], Nobutake Suzuki[1], Mai Yamagishi[1], Jun Mizuno[2], Tetsushi Sekiguchi[2], Ryuta Nishikomori[3], Shuichi Shoji[2], Osamu Ohara[1, 4]

[1]Research Center for Allergy and Immunology, Riken, Japan,
[2]Major in Nano-science and Nano-engineering, Waseda University, Japan,
[3]Department of Pediatrics, Kyoto University Graduate School of Medicine, Japan
[4]Kazusa DNA Research Institute, Japan

ABSTRACT

Live-cell imaging of inflammatory cytokine secretion from human monocytes was successfully performed on a microfabricated well array chip by time-resolved fluoroimmunoassay using total internal reflection fluorescence microscopy. Some of the lipopolysaccharide primed monocytes secreted small amounts of interleukin-1 beta (IL-1β), whereas the monocytes stimulated with an ATP signal secreted large amounts of IL-1β from 10% of observed cells. These two types of secretion patterns corresponded to the proposed models of IL-1β secretion, “rescue and redirect” and “terminal release”, respectively. Morphological changes in ATP-stimulated cells also indicated that the cells underwent necrotic cell death, also known as “pyroptosis”.

KEYWORDS

Cytokine secretion, single cell, inflammation, monocyte

INTRODUCTION

Inflammatory cytokines play a key role in the initiation of inflammation. However, their secretion dynamics, especially at the single cell resolution, still remain unclear. This is because conventional methods cannot follow the real-time secretion process at the single-cell level. For example, although the intracellular staining/enzyme-linked immunospot assay can quantify inner/secreted cytokines from a number of single cells as a snapshot of accumulated amounts, time-resolved dynamics of cytokine secretion cannot be determined by either method. Recently, some researchers developed methods, which enabled quantitative and temporal measurement of the secretion activity at single-cell resolution using microfabrication technology. Among them, Love et al. reported measurement of secretion profiles from a massive number of single cells with microfabricated well arrays [1]. They also succeeded in measurement of the time course of cytokine secretion during T-lymphocyte maturation over several hours. However, their method cannot achieve real-time monitoring of cytokine secretion, which is required to monitor more rapid phenomena during pathogenic responses of macrophages/monocytes than those during effector T-cell differentiation.

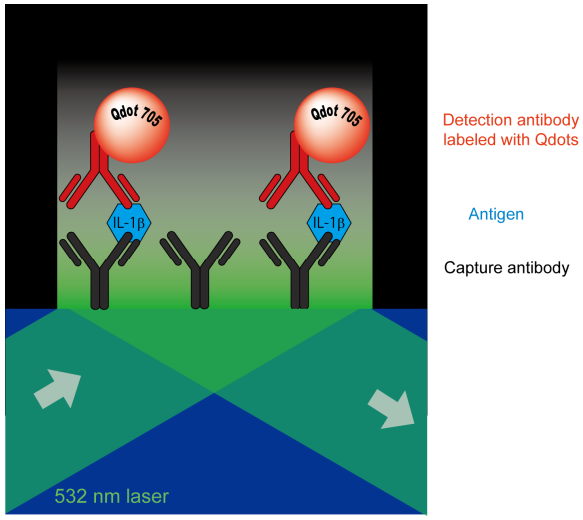

Figure 1: Scheme of observation of cytokine secretion by TR-FIA using TIRF imaging.

We previously developed a time-resolved fluoroimmunoassay (TR-FIA) from single cells by total internal reflection fluorescence (TIRF) imaging with a microfabricated well array chip [2]. This assay is based on a sandwich immunosorbent assay, but does not require time-consuming bound-free separation of detection probes. An immunocomplex consisting of capture antibody, antigen, and detection antibody on the bottom surface is observed by an evanescent field of excitation light (Figure 1). Therefore, the assay enables continuous monitoring of the secretion activity of single cells.

EXPERIMENT

Fabrication of microwell array chips

Microwell array chips were made with polydimethylsiloxane (PDMS) and a microscope slide. PDMS thin sheets, on which a 50 × 50 array of through-holes 70 μm in diameter with 100 μm center-to-center spacing were fabricated, were purchased from Fluidware Technologies Inc. (Saitama, Japan). The PDMS sheet and glass slide were air plasma treated (SEDE-PFA, Meiwafosis Co., Tokyo, Japan). They were immediately placed in the presence of methanol to eliminate distortion and were bound permanently at 130°C for 3 hours. Bare glass surfaces of the bottom of the wells were aminated with VECATBOND Reagent (SP-1800, Vector Laboratories Inc., Burlingame, CA) and set onto a cell chamber (Attofluor cell chamber, A7819, Life Technologies) with another PDMS block (Sylgard184, Dow Corning Toray Co., Ltd., Tokyo, Japan) that had a through-hole 8 mm diameter to make a tiny volume of cell reservoir (Figure 2). Capture antibodies for IL-1β were immobilized on aminated bottom glass of the

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wells via dimethyl pimelimidate in 0.1 M triethanolamine (pH 8.0) for 1 hour at room temperature. The surface was blocked with monoethanolamine (0.1 M, pH 8.2) and 0.2% wt Lipidure® reagent (NOF Co., Tokyo, Japan) and stored at 4°C until use.

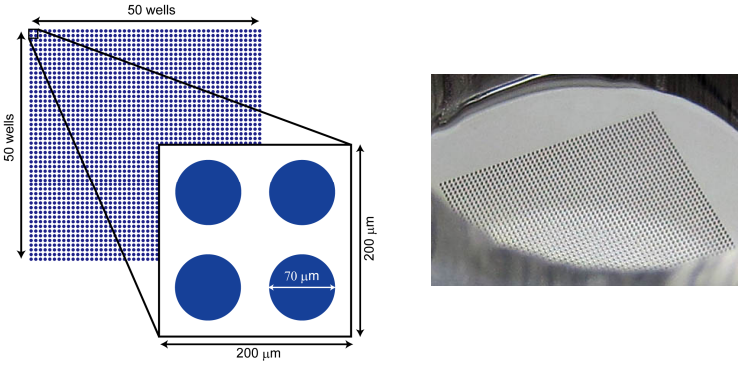

Figure 2: Schema and photograph of the microwell array chip

Cell culture

Peripheral blood mononuclear cells were obtained from peripheral blood of healthy volunteers with Lymphoprep (Axis-Shield, Oslo, Norway) according to a standard protocol from the manufacturer. CD14+ monocytes were purified by MACS (Miltenyi Biotec) with a negative selection reagent, Monocyte Isolation Kit 2, and a positive selection reagent, CD14 MicroBeads, sequentially.

Preparation of detection regents

Detection antibody labeled with biotin was conjugated with Qdot®705 labeled streptavidin (Q10161MP, Life Technologies) in a 3 to 1 molar ratio at room temperature, and light shielded for 3 hours. Unoccupied sites on streptavidin were blocked with an excess amount of $dPEG_4$-biotin acid (10199, Quanta BioDesign, Ltd., Powell, OH). Unconjugated $dPEG_4$-biotin was removed by ultrafiltration (Amicon Ultra-0.5, Merck Millipore, Billerica, MA). Detection antibody labeled with Qdot705 and albumin solution from bovine serum were added to the culture medium at a final concentration of 30 nM and 2.5% w/v, respectively. Lipopolysaccharide (LPS) was added to the detection reagent at 1 μg/mL. ATP was added to the detection reagent at 5 mM.

Optical set up

TIRF microscopy was performed on an automated inverted microscope (Eclipse Ti-E, Nikon, Japan) with a perfect focusing system with a 60× oil immersion objective (Apo TIRF, 60×, NA= 1.49). An excitation laser with a wavelength of 532 nm was reflected with a home-made incident angle controller and introduced into the microscope.

RESULTS AND DISCUSSION

We applied our system to observe IL-1β secretion from human monocytes. IL-1β protein is produced in the cytosol, on priming stimulation via Toll-like receptors or interleukin-1 receptor. It is unclear how IL-1β is secreted into the extracellular space because the molecule has no signal to be incorporated in the typical protein secretion pathway. Various features of IL-1β secretion have been reported in many studies conducted with bulk cell cultures placed under conditions where IL-1β is released into the culture supernatants. However, there is no consensus on the mechanism for IL-1β secretion because previous study results are conflicting. Therefore, four hypotheses for the mechanism of IL-1β secretion have been proposed, which are categorized along with the types of stimuli [3]. For example, Figure 3 shows two of the four proposed mechanisms of secretion. One mechanism of secreting IL-1β after weak stimulation is called “rescue and redirect”, in which IL-1β molecules in the cytosol are enveloped by autophagosomes and released after the fusion of the autophagosomes and the plasma membrane. Another mechanism, “terminal release”, is thought to occur after intense stimulation in which IL-1β molecules leak via through-holes on the plasma membrane, which is usually associated with cell death called “pyroptosis”. These mechanisms appear to explain how IL-1β travels to the extracellular space from the cytosol beyond the lipid bilayer barrier. However, it is unknown how such key events between the cytosol and the extracellular space actually occur prior to IL-1β secretion because of a lack of technology for time course imaging of secretion in single-cell resolution. Our system, which enables monitoring of the time course of cytokine secretion, proposes a solution to address the issue of IL-1β secretion.

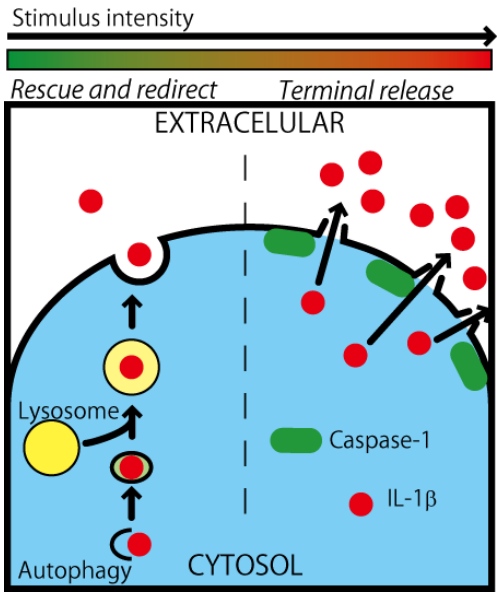

Figure 3: Proposed mechanism of IL-1β secretion.

In the current study, we attempted to compare the secretion patterns of LPS-activated human monocytes with or without additional ATP stimulation for the first time. ATP is a well-known inducer for “terminal release” of IL-1β, which induces potassium efflux and subsequently induces NLRP3 inflammasome formation. We simultaneously

observed diascopic illumination (DIA) images to confirm the location and viability of each cell. Time-lapsed images were taken in more than 1,000 wells every 25 minutes. Under the stimulation of LPS only, a few percent of cells secreted IL-1β at only low levels. Weak signals of IL-1β were observed just beneath the cells (Figure 4), which suggested that IL-1β molecules released by one emission were limited to a small amount. DIA movies showed that all the cells, including IL-1β secreting cells, moved around vigorously. However, from 20 min after ATP stimulation, approximately 10% of the cells secreted IL-1β at extremely high levels. According to the DIA images, every monocyte exhibiting a strong IL-1β signal changed their morphology to an expanded shape (Figure 5), which suggested that they were dying with loss of membrane integrity, as seen in pyroptotic cells.

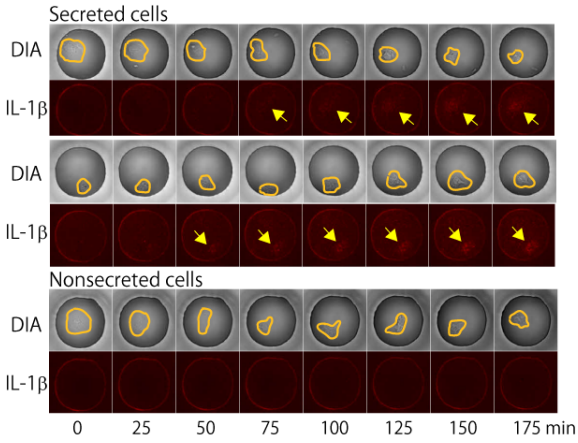

Figure 4: Observation of "rescue and redirect" of IL-1β. These micrographs show time course images of three monocytes. Two of the monocytes (upper) are weakly secreting IL-1β just beneath them. The lower panels show a nonsecreting cell. The yellow outlines show an individual cell, which is expanding on the bottom. The yellow arrows show the secreted position of IL-1β.

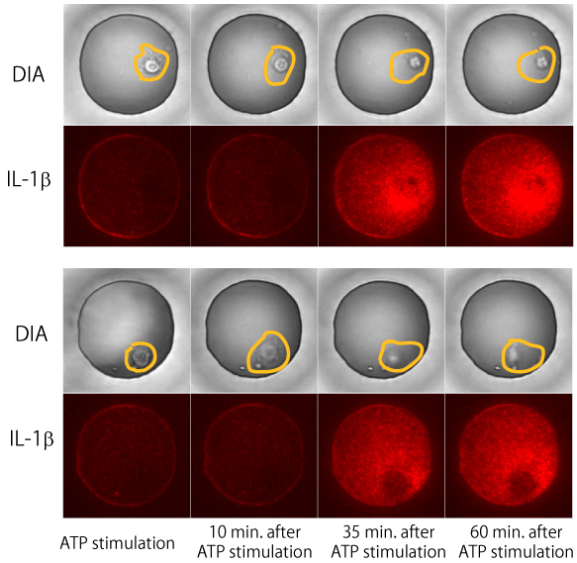

Figure 5: Observation of "terminal release" of IL-1β. These micrographs show the time course images of two monocytes. The yellow outlines show an individual cell. Nuclear condensation can be observed on the DIA image, unlike Figure 4. IL-1β signals of "terminal release" were stronger and more extensive than "rescue and redirect".

CONCLUSION

We demonstrated secretion activity from single cells in a time-dependent manner. We were able to directly monitor the secretion of IL-1β and simultaneously observe the time course in the morphology of cells for the first time. Using our system, we demonstrated that different types of stimuli actually lead to different types of IL-1β secretion. This system can be expanded by introducing other colors of probes representing other information of cell events, i.e., membrane permeability, caspase-1 activity or apoptosis, which are likely to provide more detail regarding the effect of external stimuli administration on IL-1β secretion.

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CONTACT

Yoshitaka Shirasaki +81-45-503-9696 or sirasaki@rcai.riken.jp