CHARACTERIZATION OF NATURAL KILLER CELLS' CYTOTOXIC HETEROGENEITY USING AN ARRAY OF SONO-CAGES

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ABSTRACT

Using a multi-well device as an array of sono-cages for single cell analysis, we quantify for the first time the heterogeneity of natural killer (NK) cells' cytotoxic response against cancer cells. We report a fraction of inactive NK cells within the tested population (36%), as well as the existence of few 'serial killers' that eliminate up to six targets during 4 hours. We also characterize the multi-well acoustic device in terms of trapping efficiency at different actuation voltages, using adherent and non-adherent cell lines. We show that the acoustic forces applied on the cells can be compared to forces of biological processes (*i.e.* cell adherence).

KEYWORDS: Natural killer cells**,** Cytotoxicity, Ultrasound, Multi-well, Microchip,

INTRODUCTION

NK cells are cells of the immune system that can induce cell death by the release of toxins to virus-infected cells and cancer cells. Until now, most experiments on NK cells have been performed on bulk cultures giving results that are averaged read-outs from all cells in the population. In order to identify the heterogeneity between individual cells in large populations, development of new methods for quantitative high-throughput analysis at the single cell level is needed. Here we present one such method and apply it for studying NK cell-mediated rejection of cancer (target) cells.

We have previously described the function and biocompatibility of the sono-cage platform, shown in Fig. 1 [1]. The method has been used for synchronizing ultrasound induced NK-cancer cell interactions and investigating the NK function conservation under the effect of ultrasound [2].

In this paper we quantify the trapping performance of the system for different cell types (adherent and non-adherent) and actuation voltages (0 – 10 V_{pp}), in order to confirm that the acoustic forces are in the range of natural biological forces (*e.g.*, adherence forces between cells or cells and surfaces), and thus gentle for cell manipulation. Further on, in a series of experiments we have been able for the first time to quantitatively characterize the cytotoxic heterogeneity of NK cells against cancer cells.

EXPERIMENTAL METHOD

The Device

The multi-well acoustic device is shown in Fig. 1 and is described in detail in Ref. 2.

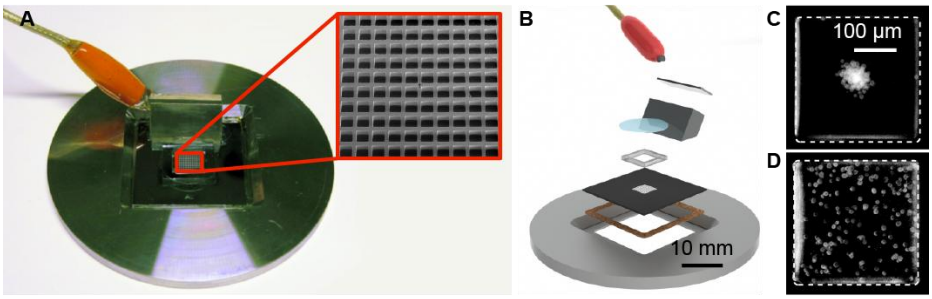

Figure 1: (A) Photo of the sono-cage platform, and a SEM picture of the array of microwells. (B) Schematic illustration of the different parts of the device (described in Refs. 1 and 2). Superposition of the 100 wells when (C) ultrasound is turned on and (D) ultrasound is turned off.

Cell culture

The cell lines used in order to investigate the trapping efficiency of our system at different actuation voltages were the 721.221 non-adherent cell line and the HEK 293T adherent cell line. 293T cells were stained and seeded in the chip together with 221 (unstained). They were allowed to settle and adhere for about one hour before imaging.

For the cytotoxic experiments, as effector cells we have used primary polyclonal human NK cells that were harvested from blood of healthy donors by centrifugation and separation of peripheral blood mononuclear cells followed by negative magnetic bead sorting. NK cells were cultured for 7 days in growth medium with the IL-2 activating factor [2].

As target cells we used the human B cell line 721.221, deficient in endogenous surface expression of major histocompatibility complex (MHC) class I proteins. The NK population was tested for purity (determined to be 99.5% pure) by flow cytometry and a high cytotoxicity was determined by a chromium release cytotoxicity assay [3]. Cytotoxic single-cell experiments were performed in parallel using the sono-cage platform.

RESULTS AND DISCUSSION

Analysis of the data obtained from the trapping efficiency experiments indicates that 90% of the non-adherent cells were trapped already at $3V_{pp}$, while a majority of the adherent cells were not trapped even at $10V_{pp}$. This supports our claim that the forces applied on the cells in our system, are comparable to the forces existing between cells and cells with surfaces.

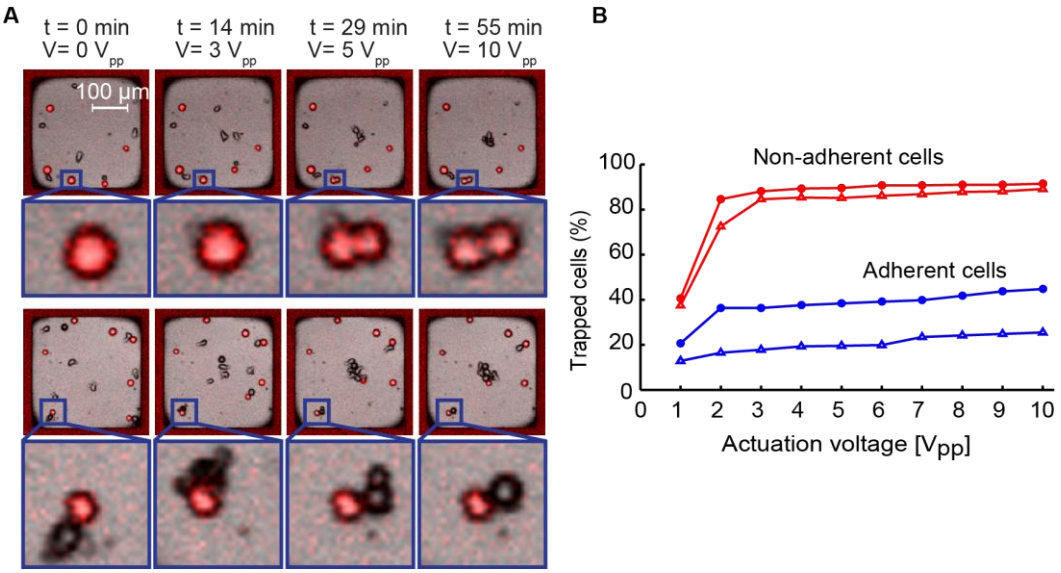

Figure 2: (A)Time-lapse of two wells containing adherent 293T cells stained with calcein-AM (shown in red) and non-adherent 221 cells (unstained) at different actuation voltages. Division of an adherent cell (upper well and zoomed in images in the second row). A 221 cell bound to an adherent 293T cell causes the aggregate to rotate and align with the acoustic force field (second well and zoomed in image in the fourth row). (B) Trapping efficiency versus actuation voltage of adherent (HEK 293T, n=279 and 255, blue lines) and non-adherent (221, n=412 and 433, red lines) cells.

For the cytotoxic experiments, human primary NK cells and target cells were stained, seeded in the chip, and then imaged four 4 hours during continuous ultrasonic actuation. The distribution of NK and target cells is shown in Fig. 3A (pooled data from four experiments). Analysis of the data revealed a significant heterogeneity in NK killing efficiency. Surprisingly, 36 % of the NK cells were completely inactive over 4 hours (Fig. 3B and Fig. 4B). The remaining 64% were active (*i.e.*, killing at least one target), but few NK cells were shown to be 'serial killers' eliminating up to six targets, as shown in Fig. 4A.

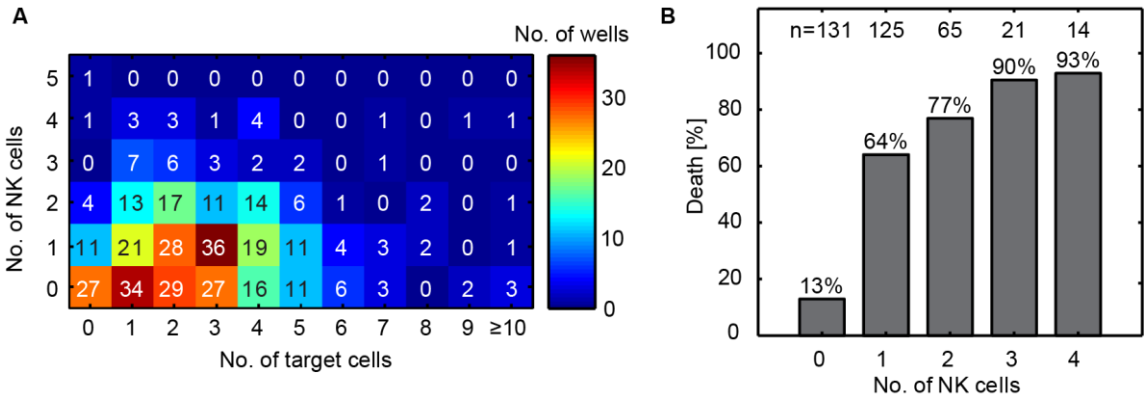

Figure 3: (A) Distribution of NKs and targets in 400 wells (summative data of four experiments). (B) The likelihood of target cell death in a 4-hour assay is increasing with the number of NK cells per well. Spontaneous death of targets occurs in 13 % of the wells. Death events are increasing to 64% in wells containing single NKs, strongly indicating NK-mediated target (cancer) cell death.

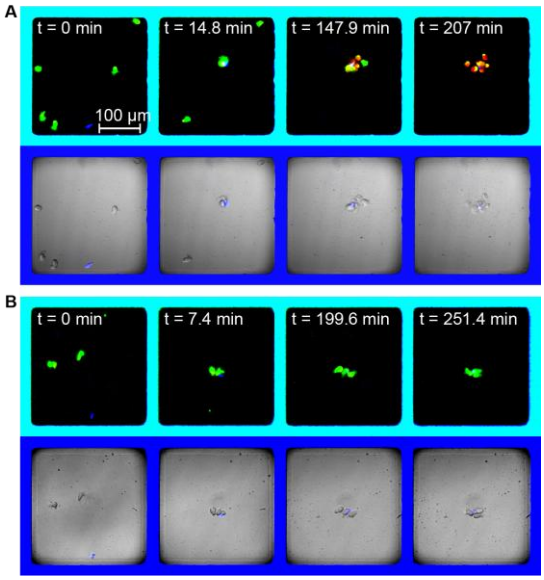

Figure 4: NKs and targets were stained and seeded in the sono-cage platform. All individual interactions were synchronized by the ultrasound actuation and time-lapse fluorescence and bright field imaging (under continuous ultrasonic actuation) was performed. Here we present: (A) A single 'serial killer' NK cell (blue cell) interacting with several live targets (green cells). At t = 207 min, all targets are dead (red cells). (B) Inactive NK cell (blue cell). All targets are still alive (green cells) after 4 hours of interaction.

We also observed a significant increase in NK killing efficiency depending on the number of targets in contact with the NK cell, as shown in Fig. 5. This dependence is justified by the increased activation of the NK cells due to the accumulated activating signal when in contact with more targets. The results are important for the development of future immuno-therapy methods.

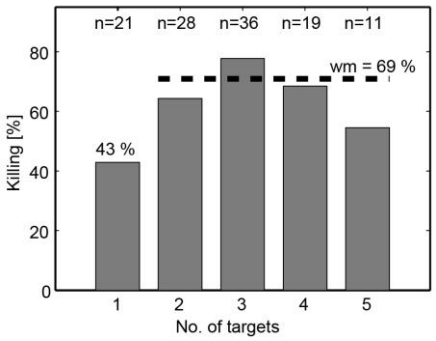

Figure 5: Wells containing single NKs and various numbers of targets were observed over 4 hours and the killing events were quantified. The results indicate that the likelihood of target cell death increases significantly when single NKs are in contact with more than one target from 43% to 69% (weighted mean value).

CONCLUSIONS

We have investigated our multi-well device in terms of trapping efficiency and we can conclude that the acoustic forces acting on cells are in the range of cell-cell interaction forces. Using our method we have shown that there is a significant heterogeneity among a NK cell population in terms of cytotoxicity against cancer cells. Furthermore, we observe that NK cells kill more efficiently when the target cell number increases from one to three targets.

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