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Lysis-on-Chip of Single Target Cells following Forced Interaction with CTLs or NK Cells on a Dielectrophoresis-Based Array

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Guiding the interaction of single cells acting as partners in heterotypic interactions (e.g., effectors and targets of immune lysis) and monitoring the outcome of these interactions are regarded as crucial biomedical achievements. In this study, taking advantage of a dielectrophoresis (DEP)-based Laboratory-on-a-chip platform (the DEPArray), we show that it is possible to generate closed DEP cages entrapping CTLs and NK cells as either single cells or clusters; reversibly immobilize a single virus-presenting or tumor cell within the chip at a selected position; move cages and their content to predetermined spatial coordinates by software-guided routing; force a cytotoxic effector to physically interact with a putative target within a secluded area by merging their respective cages; generate cages containing effector and target cells at predetermined E:T ratios; accurately assess cytotoxicity by real-time quantitation of the release kinetics of the fluorescent dye calcein from target cells (>50 lytic events may be tested simultaneously); estimate end points of calcein release within 16 min of initial E:T cell contact; simultaneously deliver Ab-based phenotyping and on-chip lysis assessment; and identify lytic and nonlytic E:T combinations and discriminate nonlytic effector phenotypes from target refractoriness to immune lysis. The proof of principle is provided that DEPArray technology, previously used to levitate and move single cells, can be used to identify highly lytic antiviral CTLs and tumor cells that are particularly refractory to NK cell lysis. These findings are of primary interest in targeted immunotherapy. The Journal of Immunology, 2013, 191: 000–000.

Laboratory-on-a-chip (Lab-on-a-chip) platforms based on dielectrophoresis (DEP) identify single cells and entrap them within single DEP cages (1–6). Cages and their content may be levitated and moved within the chip to any place on its surface (7, 8). DEP-based techniques for cell manipulation also were described, with particular emphasis on selectively positioning cells onto three-dimensional grid electrode systems (9, 10). DEP-based Lab-on-a-chip platforms are expected to levitate and move cell populations (11–13), and even single cells (7), on a scale and with performances impossible to achieve by micro-manipulation.

CTLs and NK cells form two broad classes of lytic effectors responsible for the cytolysis of susceptible target cells, including virus-infected cells and tumor cells. The simultaneous manipulation of single CTLs and NK cells on the one hand, and single target cells on the other, will substantially enhance our understanding and control of lytic interactions. Applications may be foreseen in several crucial areas of human immunology, including antiviral surveillance and tumor immunotherapy.

To the best of our knowledge, the DEP-based manipulation of single CTLs and NK cells has not been reported. Controlled, forced interaction with single target cells have never been achieved. The effects of DEP fields and conditions on the biological activity of CTLs and NK cells have not been investigated. In this study, these issues are addressed in two experimental systems: Ag-specific CTL clones differentially targeting lymphoblastoid cell lines (LCLs) pulsed with viral peptides (14) and the continuous NK cell line NKL, which lyses (among others) lymphoblastoid tumor cells lacking protective HLA class I molecules, such as HLA-G (15).

The major goals of the current study were to force single CTLs and NK cells to interact with single target cells at predetermined positions within the DEPArray, measure specific lysis in situ under DEP conditions in real time, identify effector cells with different lytic efficiencies, and identify target cells with differential susceptibilities to immune lysis.

Materials and Methods

Cell lines

LCLs for CTL studies were obtained by infection of human B lymphocytes with the B95.8 strain of EBV. The .174/T2 cell line (T2) was obtained by
fusion of the peptide transporter mutant, 174 LCL with the T cell line CEM (16). Cell lines were maintained in RPMI 1640, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Sigma-Aldrich, St. Louis, MO), and 10% heat-inactivated FCS (HyClone, Logan, UT). LCL 221 and its 221.G1 transfectants (17) were used in studies with NK cells and grown in RPMI 1640 medium supplemented with 10% FCS at 37°C in 5% CO2. The NKL continuous cell line (18) expresses the inhibitory ILT2 and CD94/NKG2A receptors specific for HLA-G and HLA-E molecules expressed by 221.G1. The latter are assembled in the presence of a specific ligand donated by the HLA-G signal sequence (15).

The DEPArray

The DEPArray prototype consists of a 8.8 mm2 chip featuring 102,400 actuation electrodes, arranged in an array of 320 × 320, 20 × 20-μm microsites, each microsite comprising addressing logic, an embedded memory for electrode programming, and an optical sensor (7, 19). The chip, which was described previously (8, 20, 21), generates spherical DEP cages, enabling software-controlled displacement of >105 individual objects, including living cells. All of the experiments described in this article were carried out at room temperature, in RPMI 1640 medium containing 10% FBS, in a sealed chip chamber, eliminating the need for CO2 injection. However, the operation of the device under controlled temperatures is also possible.

Generation of CTL cultures. Monocyte-depleted PBLs from HLA-B35 +

The percentage of specific lysis was calculated as 100 

Labeling of target and effector cells with calcein and MitoTracker Red 580. To demonstrate that a single LCL can be targeted with the desired number of selected effectors, three CTLs (CTL1, CTL2, and CTL3) were programmed to follow distinct collision routes with a single target, as indicated, whereas the fourth CTL (CTL4) present in the array was left in its initial position (Fig. 2A: brightfield; Fig. 2B, 2C: fluorescence images at time 0). A cluster was generated by targeting, in rapid succession, the immobilized LCL cell with one (D-J), two (K-N), and three (O-P) CTLs. Clips of this and a similar targeting experiment are provided as Supplemental Videos 1 and 2. After the initial set-up, which is necessary to optimize DEP conditions for each cell line, the entrapment, caging, movement, and targeting steps are highly reproducible. In a series of 1000 consecutive caging and moving attempts, we were unable to complete the intended routing in only four instances (<0.5% failure rate). It is concluded that single CTLs can be independently, sequentially, and reproducibly manipulated, and the intended clusters can be obtained within seconds.

Results

Targeting single tumor cells with a programmed number of CTLs

Conventional DEP separates two cells as long as their electrokinetic mobilities differ. In contrast, even cells with identical DEP mobilities can be caged and moved independently to different positions by the DEPArray. A typical DEPArray experiment involves a single cytotoxic immune effector and a single target cell that can be easily distinguished by cell size and morphology or, even better, by labeling the former with the fluorescent mitochondrial dye MitoTracker Red 580 and the latter with the green fluorescent cytoplasmic dye calcein (Fig. 1A). The immune effector is entrapped at its initial position by forming a DEP cage. Then, it is moved under microscopic control within the chip toward the target, which is caged and held in place at a different grid position (Fig. 1B). A programmed, forced interaction is achieved at this predetermined position upon fusion of the DEP cages (Fig. 1C), and target cell lysis is monitored in real time by observing the loss of green fluorescence (Fig. 1D).

FIGURE 1. Step-wise targeting of a single LCL with a single CTL. A single CTL labeled with the red fluorescent dye MitoTracker Red 580 is routed (A) and targeted (B) to a single cell labeled with green calcein (left panels). Following forced interaction (C), target cell lysis is visualized by calcein loss (D). Bright-field and fluorescence images of the actual experiment are recorded by the built-in microscopic apparatus of the DEPArray (right panels). Images of fluorescent cells were recorded during the very early phases of the experiment lasting <1 min.
200 target LCLs in four independent experiments. LCLs were DEP caged (individually and in clusters) at specific array positions and observed by fluorescence microscopy. Minor, if any, decreases in calcein fluorescence were apparent, as shown by representative photomicrographs and a quantitative analysis performed over a period of 20 min (Fig. 3A, 3B). Target cells remained viable in the DEPArray for much longer times; in independent experiments, ~90% of the tested cells retained calcein for \( \geq 60 \) min (Supplemental Table I). Accordingly, a standard 5-h cytotoxicity assay did not detect significant differences in \(^{51}\text{Cr}\)-release (either spontaneous or CTL mediated) between single-labeled (\(^{51}\text{Cr}\) alone) and double-labeled (calcein and \(^{51}\text{Cr}\)) LCL target cells at E:T ratios of 5:1 (data not shown) and 15:1 (Fig. 3C). Thus, DEP buffers and manipulation have a negligible effect on spontaneous calcein release for the first 20 min of observation and a minor effect at 60 min, with 20 min representing a stringent and conservative time window in which to conduct cytotoxicity experiments.

Next, specific immune lysis was monitored and quantitated in the DEPArray. LCLs capable of presenting an EBNA1 epitope in the context of HLA-B35 (14) were Ag pulsed, calcein labeled, and caged in the DEPArray, with each LCL target having four CTLs (Fig. 4A) from an HLA-B35–restricted, EBNA1-specific T cell clone, prepared as described in Materials and Methods. Microscopic images of a representative cluster (Fig. 4B) and the kinetics of calcein release from six individual clusters (Fig. 4C) demonstrate rapid immune lysis, which is evident at \( \leq 14 \) min (i.e., well within the conservative 20-min observation window). Although calcein release is rapid in the DEPArray format, its end point is in agreement with the extent of \(^{51}\text{Cr}\)-release measured in parallel by a bulk 5-h cytotoxicity assay run at high E:T ratios with the same preparations of CTLs and target cells (Fig. 4D). In contrast, Ag-pulsed HLA-B35– cells (predicted to be resistant to lysis) released very small amounts of both calcein and \(^{51}\text{Cr}\) when either clustered with four CTLs or tested in bulk under identical conditions (Fig. 4E–H), further demonstrating the specificity of DEPArray assessments. Altogether, it may be concluded that the DEPArray and the \(^{51}\text{Cr}\)-release assay provide similar information; however, the former is much faster and reveals subtle cluster-to-cluster differences in the kinetics of calcein release (Fig. 4C, 4G) that would be undetectable in bulk cytotoxicity assays (Fig. 4D, 4H).

Because assessing cytotoxicity at the single cell level is a crucial, open issue in immunology, we sought to determine whether poor lysis depends on poor effector performance or target refractoriness to lysis. To address this issue, an approach (called “preclustering”) was specifically designed. In this approach, clusters of target cells and CTLs are not formed by forcing predetermined numbers of target and effector cells into the same DEP cage, instead they are formed by preparing a cell suspension containing CTLs and targets at a 10:1 E:T ratio in a test tube, dispensing it in the

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**FIGURE 2.** Sequential targeting of a single LCL with selected CTLs. Light microscopy (A) and fluorescent (B, C, lower magnification) images show MitoTracker Red 580–labeled CTLs (red arrows) being sequentially moved (software-programmed routes are represented by blue arrowed lines) toward a single target cell labeled with calcein (green arrow). Pictures of CTLs along their routes and stepwise clustering of the target cell with one, two, and all three CTLs are provided in (D–J), (K–N), and (O and P), respectively. A corresponding video is provided (Supplemental Video 1).

**FIGURE 3.** DEP manipulation induces negligible changes in membrane permeability of lymphoid cells. Lymphoid cells were loaded with calcein and then arrayed as either single cells (A, upper panel) or clusters of four to six cells (A, lower panels). (B) Fluorescence intensity was monitored by taking photographs at time 0 and every 2 min for 20 min and quantitated by on-chip sensors. Note that in some cases the relative positions of the cells entrapped in the same cage may differ at time 0 and at 20 min as the result of cell rolling (exemplified in A, lower panel). However, clusters are stably caged and do not dissociate. (C) Target cells, either single labeled with \(^{51}\text{Cr}\) or double labeled with \(^{51}\text{Cr}\) and calcein, were tested in a standard 5-h \(^{51}\text{Cr}\)-release assay at a 15:1 E:T ratio with Ag-specific polyclonal CTLs to the HPV Ag of EBV. Nonspecific (spontaneous \(^{51}\text{Cr}\)-release) lysis and CTL-specific lysis are represented by the white and black bars, respectively.
FIGURE 4. The DEPArray discriminates targets that are susceptible and resistant to lysis by CTLs. T cells from a specific CTL clone were forced to form clusters with a single peptide-pulsed, calcine-labeled, HLA-B35+ (A), and an HLA-B35− (E) target cell. Black arrows point to the visible CTLs. Calcine release (lysis) was monitored at 2-min intervals for 14 min by fluorescent microscopy [two representative target cells are shown in (B) and (F)] and quantitated in six HLA-B35+ targets (susceptible to lysis) (C) and six HLA-B35− targets (resistant to lysis) (G). (D and H) The same cells were tested at the indicated E:T ratios in a 5-h [51Cr]-release assay. Black and white bars depict specific and nonspecific (target-only) lysis.

DEPArray, and then applying DEP migration forces exclusively with the aim of quickly selecting and distributing, among randomly preformed clusters, those containing ~10 cells. Ordered patterns can be formed by this means, as exemplified in Fig. 5A (top panels, bright-field), and immune lysis can be monitored in each cluster (middle and bottom panels). Although target cells cannot be discerned easily in bright-field images because they are outnumbered and hidden by effectors, fluorescent cell counts at time 0 (right after patterning) are consistent with each cluster containing from zero to two target cells (middle panels), as expected, with the remaining cells being CTLs. Therefore, even assuming that a fraction of CTLs in a given cluster is inactive, the E:T clustering ratio is high enough to ensure the presence of at least some lytic CTLs and one fluorescent target in most clusters. Under these conditions, the absence of lysis is likely due to intrinsic refractoriness of the target.

In agreement with this prediction, calcine was released from most (5/8), but not all, of the HLA-B35+ targets (putative susceptible) and from none (0/8) of the HLA-B35− targets (putative resistant) seen in a representative array area (Fig. 5A, compare left and right panels). A detailed time-course of calcine release on 40 targets (Fig. 5B) and a [51Cr]-release assay conducted in parallel on the same cell populations (Fig. 5C) at the optimal assay end points of 16 min and 4 h, respectively, are consistent with refractoriness to lysis being a specific property of a minority (~15%) of putative-sensitive target cells. A clip comparing calcine release in clusters containing HLA-B35+ and HLA-B35− cells is provided (Supplemental Video 3).

To improve throughput, readability, and immediacy of the pre-clustering test, HLA-B35+ target cells were peptide pulsed and double labeled using calcine and the integral fluorescent dye MitoTracker Red 580, which is not significantly released upon cell lysis. Fig. 5D shows a preclustering experiment involving 55 patterned, double-fluorescent CTL:LCL clusters. As shown in four representative clusters (Fig. 5E–H), all of the target cells remained red until the end of the experiment (although some variability was observed), whereas the extent and kinetics of calcine release differed greatly. Remarkably, in the minority of CTL:target cell clusters containing two fluorescent target cells, we observed lysis of both targets (data not shown) and selective lysis of one target cell only (see the representative example in Fig. 5I), providing conclusive evidence that the DEPArray discriminates single cells refractory to lysis among putative-susceptible targets. Thus, the DEPArray probes individual targets in individual clusters at a high multiplicity of T cell effectors, directly identifying target cells that are refractory to immune lysis. This task cannot be accomplished with any of the available bulk-testing cytoxicity techniques.

**Lysis on the DEPArray of target cells upon forced cellular interactions with NK cells**

Next, we tested the DEPArray in an NK cell system. Whereas CTLs are triggered by a clonotypic rearranging receptor that recognizes a unique Ag presented in the context of a specific class I HLA allotype (nonself), NK cells are inhibited by nonrearranging receptors with coarse specificity that sense the presence on the target of a variety of ligands, including broad groups of class I HLA molecules. Transfection of the nonclassical class I HLA-G molecule engages the inhibitory NKG2A and ILT2 receptors expressed by the continuous NK cell line NKL (18), protecting a susceptible target (LCL 221) from lysis (15, 17). Like CTLs, NK cells could be moved in the DEPArray and caged with a parental 221 target cell (Fig. 6A). Clusters were then formed that contained two to five NKL cells with one target cell, either a parental 221 cell or an HLA-G+ 221.G1 transfectant (Fig. 6B, light microscopy). As expected, 6/9 of the former and only 1/9 of the latter were susceptible to lysis (see representative clusters in Fig. 6B) and the kinetics of calcine release in Fig. 6C), demonstrating specific lysis. Thus, similar to CTLs, occasional resistance and susceptibility to NK cell lysis was observed in putative-susceptible and putative-resistant target cell populations, respectively. Altogether, DEPArray results were in agreement with a [51Cr]-release assay performed in parallel (Fig. 6D).

To demonstrate that the DEPArray discriminates susceptibility and resistance to NK cell lysis on the single-cell level, equal numbers of calcine-loaded 221 and 221.G1 target cells were admixed and simultaneously assessed for NK lysis and HLA-G expression.
FIGURE 5. Preclustering experiments. Peptide-pulsed, HLA-B35<sup>+</sup> (putative-susceptible to lysis) and HLA-B35<sup>−</sup> (putative-refractory to lysis) LCLs were calcein labeled and resuspended in a test tube with CTLs (E:T ratio = 10:1). (A) The cell suspensions were dispensed in the DEPArray, and preformed spontaneous clusters containing ~10 cells were DEP patterned. Fluorescence intensity of individual target cells in each cluster was analyzed at the indicated times. (B) Plot of individual determinations from 40 target cells (average ± SD). ●, HLA-B35<sup>+</sup> LCL cells; ○, B35<sup>−</sup> LCL cells. A video of this experiment is provided (Supplemental Video 3). (C) The same cell suspensions used in (A) and (B) were tested for specific and spontaneous (no effector) [51Cr]-release (black and white bars, respectively). (D) Target LCLs (putative susceptible, prepared as above) were double labeled with calcein and MitoTracker Red 580, preclustered with specific CTLs, and dispensed in the DEPArray as above. Preformed clusters (n = 55) were quickly patterned, and the green and red fluorescence intensities were recorded at 0, 8, and 16 min. (E–H) Fluorescence microscopy images of the four representative clusters (color-coded) shown in (D). (I) A bright-field image and two fluorescence microscopy images (at time 0 and 12 min) depicting a single cluster containing two fluorescent target cells (arrows). Only the target cell to the left (arrow missing at 12 min) was susceptible to CTL lysis.

Discussion

To the best of our knowledge, this is the first report of software-programmed movement and interaction of single CTLs and single NK cells with single target cells. We show that a novel DEP-based Lab-on-a-chip platform effectively manipulates immune effectors and their targets. We describe a real-time method to visualize lysis by monitoring the release of a green fluorescent dye. Green fluorescence may be normalized (if necessary) by counterstain with a nonreleasable red dye. We demonstrate the compatibility of our approach with target cell phenotyping by microspheres coated with mAbs and provide the proof of principle that refractory targets, as well as nonlytic effectors, exist within phenotypically homogeneous cell populations.

Several approaches (24–33) have been described to assess immune lysis, including the classical [51Cr]-release assay (26, 27), the europium (Eu3<sup>+</sup>)-release assay (28–30), BLT esterase activity (31), β-galactosidase release (32), FACS assays (33), and video microscopy (34, 35). Particularly relevant to the current study, a microfabricated device has been described (36) and is commercially available (xCELLigence; http://www.aceabio.com/case_info.aspx?id=249), enabling the dynamic, real-time, label-free detection of NK cell lysis by measurement of electric impedance in a microtiter format.

Notwithstanding the advantages of each approach, only the presently described DEPArray integrates the single-cell resolution typical of videomicroscopy with the ability to individually and simultaneously monitor a significant number of lytic events, as required for cell-population analysis. High-resolution (confocal) microscopy and videomicroscopy have been used in most cases to describe changes occurring within seconds during the formation of the immunological synapse (e.g., in qualitative, single-cell experiments in which throughput is a minor concern and the observation of individual lytic events depends on random formation within the DEPArray. To avoid coating the entire surface of 221. G1 cells with Abs that might significantly interfere with NK receptor engagement, the cell suspension was incubated with red fluorescent beads coated with an Ab to HLA-G. As expected, approximately half of the cells formed rosettes (data not shown). When the cell suspension was dispensed into the DEPArray, protection from lysis and expression of the protective HLA-G molecule (rosetting) correlated, as seen in the representative example (Fig. 6E–J). Rosetting cells did not appreciably lose fluorescence for ≥12 min in the presence of NK cells (Fig. 6K), demonstrating efficient protection. Thus, the DEPArray identifies cells refractory to NK cell lysis in a mixed population of NK cell-sensitive and NK cell-susceptible targets.

In summary, the results in Figs. 5 and 6 clearly show that the DEPArray identifies, within heterogeneous populations, individual effectors and individual targets that are poorly cytotoxic and highly refractory to immune lysis, respectively. This provides the proof of principle that the DEPArray appreciates the complexity of the lytic interactions entertained by the two major classes of immune effectors and their targets.
of cell–cell contacts within the microscopic field). In contrast, the DEPArray has been designed for low-resolution microscopy (10× or 20× optical magnification) and medium-throughput analysis of killing kinetics. The DEPArray has the exquisite ability to manipulate and move hundreds of individual cells in parallel and at will. The interaction partners can be selected even when seeded at distant positions and when belonging to complex cell populations (as long as individual populations can be identified by size, morphology, and/or phenotyping with specific Abs, as shown in this study). Single cells can be sequentially targeted, and lytic events are precisely timed from their controlled onset to a convenient end point.

Immunologists often rely on surrogate “activation” markers (e.g., lymphokine secretion or expression of surface molecules, such as CD107a) to indirectly estimate cytolysis. The specificity and potential lytic ability of CTLs is routinely assessed by ELISPOT and T cell binding of multimeric HLA class I molecules, and potential lytic ability of CTLs is routinely assessed by ELISPOT and T cell binding of multimeric HLA class I molecules, and potential lytic ability of CTLs is routinely assessed by ELISPOT and T cell binding of multimeric HLA class I molecules, and potential lytic ability of CTLs is routinely assessed by ELISPOT and T cell binding of multimeric HLA class I molecules, and potential lytic ability of CTLs is routinely assessed by ELISPOT and T cell binding of multimeric HLA class I molecules. The DEPArray outperforms these traditional assays, because it provides a straightforward measurement of cell lysis (e.g., the ultimate cytotoxicity readout in vitro, regardless of surrogate or indirect “activation” markers).

Although the throughput of flow cytometry and other “bulk” cytotoxicity techniques, such as the standard isotopic (⁵¹Cr) and Eu³⁺-release assays, is considerably greater than that of the presently described DEPArray platform, we show in this article that calcein- and chromium-release assays are in substantial agreement on the whole. In addition, a DEPArray kinetic analysis of lysis in multiple cellular clusters describes lytic interactions at an unprecedented resolution. Moreover, flow cytometry identifies several thousands of individual lytic events, but unlike the DEPArray, it fails to identify the effector(s) responsible for each of them.

Throughput may represent a significant limitation of the tested prototypal DEPArray version in certain experimental settings. To leave enough space for routing, no more than 4000 DEP cages can be filled with objects (either effector or target cells). Commercially available systems (http://www.siliconbiosystems.com) have extended these limits to 40,000 independently routable cages. To improve throughput to an extent that would make the assay useful to test nonclonal target and effector cells obtained from live organisms, the preliminary enrichment of CTL and NK cell populations may be required, by either Ab or tetramer staining (38, 39), followed by sorting (40). This exemplifies the complementary nature of DEPArray and classical techniques.

Nonspecific isotope release from target cells is a common, unavoidable source of “background” in standard cytotoxicity assays reported by many groups (41–43) and is routinely taken into account when calculating lytic performance and specificity. Typically, specific lysis is estimated by subtracting “spontaneous” isotope release (e.g., in the absence of effectors) over a 4–5-h incubation time. In contrast, the few cells that may have released calcein without contact with a specific effector are excluded ex ante from DEPArray testing, facilitating analysis and improving accuracy. Additional practical advantages of the DEPArray are the nonradioactive format and the rapid completion (minutes) time. This is likely due to the fact that lysis does not depend on slow, random formation of E:T contacts in suspension, but heterotypic partners...
are forced to interact, shortening the time required to observe the lytic outcome.

It is now possible to answer several questions in the field of applied immunology. In the traditional bulk assays, cytotoxicity is highest at the highest E:T ratios. Although the DEPArray identifies single CTLs lysing single target cells, the highest levels of cytotoxicity require clusters formed in conditions of effector excess (i.e., in conditions that mimic the traditional chromium-release assay). In bulk cytotoxicity assays, several mechanisms can account for the requirement of high E:T ratios (e.g., multiple successive targeting by the same or different effectors, often referred to as recycling) or increased collision rates when effectors and targets are seeded at high densities. By providing complete control over these variables, the DEPArray format directly demonstrates that immunolysis, at least as assessed by in vitro assays, requires the synergy among multiple effectors while bound to a single target. Although the requirement for synergy precludes a systematic analysis of individual E:T interactions, the preclustering approach provides evidence for populations of clonally derived CTLs differing in the times required to lyse susceptible targets (i.e., there is heterogeneity among CTLs), and the DEPArray accurately measures this heterogeneity. Likewise, clusters may contain refractory and susceptible targets, as best exemplified in NK cell experiments. Therefore, the DEPArray is of potential interest to identify highly cytotoxic CTLs/NK cells and highly refractory targets. This issue may be of relevance in monitoring CTL-based immunotherapy (37).

Viruses and tumors escape CTLs and NK cells by reducing and increasing the expression of activating and inhibitory ligands, respectively, expressed on target cells (44). HLA-G is expressed in several tumors (45). Therefore, the DEPArray offers the possibility to detect infected cells and tumors that inappropriately express known and unknown immune ligands.

Although this report is focused on a very limited set of cell–cell interactions of immunological interest, the DEPArray also offers the opportunity to study the induction of complex phenotypic traits (e.g., changes in morphology, adhesion, motility, and differentiation) arising following the guided interaction between different types of nonimmune cells, including stem cells from body fluids and tissues. Hypothetical future applications include cancer (immuno)profiling, prenatal diagnosis, and regenerative medicine.

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Disclosures
N.M. and G.M. hold leadership positions in and are shareholders in Silicon Biosystems. M.T. and R. Guerrieri are shareholders in Silicon Biosystems. The other authors have no financial conflicts of interest.

References


