Single-Cell Technologies for Monitoring Interactions Between Immune Cells

by

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B.S.E., Biomedical Engineering, Duke University (2008)

Submitted to the Department of Biological Engineering in partial fulfillment of the requirements for the degree of

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Abstract

Immune cells participate in dynamic cellular interactions that play a critical role in the defense against pathogens and the destruction of malignant cells. The vast heterogeneity of immune cells motivates the study of these interactions at the single-cell level. In this thesis, we present new tools to characterize how individual immune cells interact with each other and with diseased cells.

We develop a nanowell-based platform to investigate how natural killer (NK) cells interrogate and attack diseased target cells. This platform enables integrated analysis of cytolytic activity, secretory activity, receptor expression, and dynamic parameters of interactions between thousands of individual NK cells and target cells. Using this platform, we show that NK cells operate independently when lysing a single target and that motility during contact is associated with the secretion of certain cytokines. Extending the platform, we investigate how contact with a target induces the shedding of CD16 from the surface of an NK cell. NK cells use CD16 to recognize antibody-coated target cells, and thus the loss of CD16 is of clinical interest. We show that the loss of CD16 is correlated to the length of time that the NK cell spends in contact with a target but that not all NK cells that shed CD16 exert common effector functions. In wells with multiple NK cells, shedding occurs in a more coordinated manner than would be expected by chance alone. Next, we compare the functional properties of NK cells with distinct repertoires of inhibitory receptors. Inhibitory receptors prevent NK cells from attacking healthy cells, and their expression can confer NK cells with increased functional activity in a process known as “licensing”. We show that despite forming prolonged contacts with target cells, unlicensed NK cells are less likely than licensed NK cells to secrete cytokines. Finally, we present tools to study other modes of interaction between immune cells. Towards this end, we develop and apply fluorescent cellular barcoding strategies to efficiently analyze the secretory properties of individual immune cells from different populations. Altogether, this thesis contributes new tools for single-cell analysis and applies them to reveal new insights about intercellular interactions.
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Chapter 1

Introduction: Single-Cell Measurements in Immunology

Portions of this chapter are adapted from Ref. 1 and Ref. 2.


1.1 Importance of single-cell measurements in understanding immune responses

Attempting to dissect cellular immune responses presents a conundrum: On the one hand, the vast heterogeneity of immune cells means that we need to characterize each individual cell with a high level of detail; on the other hand, the dense interconnectedness of the immune network means that we must also assess how cells interact with and are influenced by the cellular community as a whole. In this thesis, we develop and apply tools to characterize natural killer (NK) cells and T cells, which are two types of immune cell that epitomize this conundrum due to the fact that they are extremely heterogeneous, polyfunctional, dynamic, and involved in many types of cellular interactions.

Heterogeneity

One of the most striking examples of heterogeneity in the immune system comes from the rearrangement of T cell receptor (TCR) gene segments to generate receptors capable of recognizing a diverse assortment of antigens. It is estimated that the lower limit of each person’s TCR repertoire is on the order of $10^6$ clonotypes,\(^3\) which means that typically only one in $10^3$ – $10^5$ T cells will recognize any given antigen.\(^4\) Similarly, although NK cells do not express antigen-specific receptors that are generated by gene rearrangement, they express a variety of germline-encoded surface receptors that allow them to differentiate between diseased target cells and healthy host cells.\(^5\) The expression of these receptors varies not only between individuals, but also between NK cells within the same individual. This variability is partly due to the stochastic gene expression of certain receptors.\(^6\)

In addition, a substantial amount of heterogeneity in populations of T cells and NK cells exists even when specific gene rearrangements or stochastic receptor expression are ignored. Both T cells and NK cells can be subdivided into many different—but often overlapping—classes that reflect differences in function, prior history, tissue localization, and state of differentiation and activation. For example, studies char-
acterizing a large number of surface receptors and cytokines expressed by individual cells have shown that CD8$^+$ T cells populate a continuum of intermediary states between major states, such as naïve and memory, and that even highly specified subsets of T cells exhibit extensive combinatorial diversity in their expression of cytokines.\(^7\) Multiparameter characterizations of receptors on NK cells have demonstrated that these cells also exhibit extensive combinatorial diversity—currently, it is estimated that each person carries 6,000 – 30,000 distinct subsets of NK cells.\(^8\) Thus, technologies are needed that enable the analysis of large numbers of individual cells in an efficient manner.

**Polyfunctionality**

Immune cells are multitalented—like precocious students, they are able to participate in many different activities, often at the same time. One form of polyfunctionality arises when a single cell performs two (or more) distinct types of function. For example, CD8$^+$ cytotoxic T lymphocytes (CTLs) and NK cells mediate two distinct, hallmark functions: the cytolysis of target cells and the secretion of cytokines. Depending on the setting and the specific cells and cytokines analyzed, these two functions can occur discordantly\(^9\) or with varying degrees of overlap\(^10-12\) within each cell. Thus, technologies are needed that enable the integrated analysis of multiple types of functions performed by the same individual cell.

Polyfunctionality can also arise due to the fact that within one class of function, it is possible to have many different “flavors”. For example, a single T cell or NK cell can secrete a wide range of different cytokines, each with different downstream effects. In T cells, this form of secretory polyfunctionality is associated with protective immune responses against pathogens (discussed in Section 1.2.3). Thus, technologies are needed that enable multiplexed analysis of cellular functions.

**Dynamic properties**

Both T cells and NK cells transition through a continuum of different phenotypic and functional states as they develop, encounter antigen or other stimuli, prolifer-
ate, home to sites in the periphery, mediate effector functions, and possibly form long-lived populations of memory\textsuperscript{1} cells. In addition, even cellular subsets that were initially thought to represent stable lineages (e.g., classes of CD4\textsuperscript{+} T helper cells) are increasingly being recognized as having plastic, flexible characteristics.\textsuperscript{14} These slowly evolving transitions, which occur over days or longer, highlight the need for technologies to monitor—or infer—cellular histories spanning long periods of time.

Dynamic events that occur over shorter time frames are also of great importance in immunology. Upon discrete stimulation (for example, a T cell meeting an antigen-presenting cell (APC), or an NK cell meeting a target cell), intracellular signaling responses can propagate in seconds, triggering dynamic outcomes that evolve over minutes to hours. These outcomes include changes in motility, the formation of a synapse, cytolysis, and the secretion of cytokines. Thus, technologies are needed that monitor dynamic properties of single cells with a temporal resolution that is sufficient to capture transitions of interest.

### Cellular interactions

Immune cells do not lead hermetic lives; rather, they interact with each other and with other types of cells. These interactions can take place through direct contact or through intermediaries such as secreted cytokines.

Both T cells and NK cells are highly motile. They migrate around lymphoid and peripheral tissues collecting and interpreting signals that are presented on the surfaces of other cells. This process is mediated by direct cell-cell contacts that can be either transient (immunological kinapses) or stable (immunological synapses).\textsuperscript{15} In the case of a naïve T cell interacting with an APC displaying cognate peptide on major histocompatibility (MHC) complexes, the signaling that results from the interaction can activate the T cell and induce proliferation, synthesis of cytokines and effector molecules, and differentiation into effector or memory subsets. In the case of a CTL or NK cell interacting with a target cell, the synapse or kinapse can serve as a conduit through which cytotoxic granules containing perforin and granzyme are transferred.

\textsuperscript{1}Certainly in the case of T cells, and possibly for NK cells\textsuperscript{13} as well.
from the CTL or NK cell to the target cell.\textsuperscript{16} Thus, to investigate processes such as the activation of antigen-specific T cells or the cytolysis of target cells, technologies are needed that monitor how cells respond to discrete, contact-mediated interactions with other cells.

In addition to cellular interactions arising from direct physical contact, immune cells communicate with each other and with non-immune cells by secreting soluble factors such as cytokines. This route of communication forms a dense network (where cells are nodes and cytokines are edges) that has similarities to human social networks.\textsuperscript{17} T cells and NK cells use secreted proteins to attract other cells to the site (by the secretion of small cytokines known as chemokines), bias CD4\textsuperscript{+} T helper cells towards different lineages, induce or inhibit proliferation, and control the balance between inflammation and immunosuppression. Secreted cytokines can also directly modulate the susceptibility of cells to pathogens. For example, interferon-\(\gamma\) (IFN-\(\gamma\)) inhibits viral replication in infected cells and enhances the microbicidal activity of macrophages,\textsuperscript{18} and macrophage inflammatory protein-1\(\beta\) (MIP-1\(\beta\), or CC chemokine ligand 4 (CCL4)) suppresses human immunodeficiency virus (HIV) infection by inhibiting the binding of HIV to a co-receptor on susceptible cells.\textsuperscript{19,20} These examples highlight just a few of the ways that immune cells use secreted factors to influence each other and to drive disease outcomes. Because immune cells display heterogeneous and dynamic secretory properties, technologies are needed that measure the secretory profiles of individual cells (or small groups of cells) over time.

### 1.2 Technologies for analyzing individual immune cells

Here, we discuss both established and recently developed techniques for analyzing the phenotypic, functional, and transcriptional properties of large numbers of individual immune cells, with a focus on T cells and NK cells.
1.2.1 Flow cytometry

To date, flow cytometry has been the method of choice for creating single-cell phenotypic profiles of immune cell populations. Flow cytometry enables rapid quantification of \(~\sim 17\) analytes (surface markers, intracellular proteins, labeling dyes, etc.) on or in single cells.\(^{21}\) The surface-marker phenotypes of immune cells are commonly measured by flow cytometry after staining the cells with fluorophore-conjugated antibodies that are specific for the markers of interest. Intracellular proteins can be assessed by fixing and permeabilizing cells prior to staining and analysis. This technique has been applied to measure the phosphorylation status of intracellular proteins (“phospho-flow”),\(^{22}\) providing rich sets of data with which to construct intracellular signaling networks in T cells.\(^{23}\) Cellular production of cytokines can also be assessed by flow cytometry using a technique, termed intracellular cytokine staining (ICS), in which cells are treated with pharmacological inhibitors of secretion so that cytokines that would normally be secreted are instead associated with the cell (and hence with the signal measured by the flow cytometer).\(^{24}\) In addition to these applications, flow cytometry is a useful tool for characterizing the proliferative and cytolytic capacities of individual cells, discussed below.

Proliferation

The proliferation of individual immune cells can be characterized using a variety of flow cytometric approaches.\(^{25}\) Flow cytometry-based proliferation assays can be combined with simultaneous surface marker and intracellular cytokine staining to generate integrated measurements of proliferation, phenotype, and cytokine production from single cells. A classic method for identification of actively proliferating cells is incorporation of thymidine analogues such as 5-bromo-2'-deoxyuridine (BrDU) or 5-ethynyl-2'-deoxyuridine (EdU) into newly synthesized DNA.\(^{26,27}\) The incorporated analogues are stained with fluorescently labeled antibodies (for BrDU) or azides (for EdU) and the resulting fluorescence signal is read out on a flow cytometer to identify cells with newly synthesized DNA. One drawback of BrDU and EdU labeling is
that cells must be fixed and permeabilized prior to data acquisition, and thus cannot be repeatedly observed over multiple rounds of division or recovered for downstream analysis.

Several types of stably incorporated live-cell fluorescent dyes have been developed that allow proliferation measurements to be made on viable cells. A parent population of cells is labeled with the dye, and upon each round of division the dye content is split equally between the two daughter cells. The division history of individual cells is inferred based on the relative intensity of dye staining measured by flow cytometry. Cells from the parent population are brightest, whereas cells from subsequent generations are dimmer due to the dilution of dye over successive rounds of division. Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a commonly used dye for such assays, and can track up to eight rounds of division.\textsuperscript{28}

\textbf{Cytolytic outcomes}

The death of individual target cells can be identified with flow cytometry using fluorescent stains against different markers of cell death. Cells undergoing apoptosis are identified by annexin V, which labels phosphatidylserine that translocates to the outer cell membrane early during an apoptotic event.\textsuperscript{29} Cells that have undergone nuclear fragmentation are identified by membrane-impermeant DNA intercalating dyes such as propidium iodide (PI),\textsuperscript{29} 7-Amino-Actinomycin D (7-AAD),\textsuperscript{30} or SYTOX.\textsuperscript{31} One limitation of traditional dead cell markers is that they do not distinguish between target cells that died in a cytolytic interaction and target cells that died from other causes. This limitation can be addressed by using fluorogenic caspase\textsuperscript{32} or granzyme B\textsuperscript{33} substrates to more specifically label target cells that have received a lytic hit from an effector cell.

The lytic potential of individual effector cells is commonly monitored using indirect readouts of cytolytic activity. Surface expression of the degranulation marker CD107a on effector cells can be conveniently detected using flow cytometry and has been shown to correlate with target cell death.\textsuperscript{11} However, stimulated effector cells that have not been involved in a cytolytic interaction can also degranulate and express surface
CD107a, limiting the specificity of the readout under certain conditions. Conjugates between effector and target cells can be assessed by flow cytometry,\textsuperscript{30} although this strategy cannot be used to identify target and effector cells that interacted but then dissociated prior to the data acquisition.

### 1.2.2 Cytometry by time-of-flight (CyTOF)

In traditional flow cytometry, spectral overlap between the fluorescent reagents that are used to label cellular markers imposes an upper limit on the number of analytes that can be simultaneously resolved (typically <20 analytes per cell.\textsuperscript{21,34} To increase the depth of profiling, a cytometry technique has been developed that uses mass spectrometry to analyze single cells that have been labeled with antibodies that are conjugated to elemental (metal) tags instead of fluorophores.\textsuperscript{35,36} This method, called mass cytometry or cytometry by time-of-flight (CyTOF), eliminates the problem of spectral overlap and therefore enables the simultaneous measurement of >36 analytes per cell (in theory, \(\sim\)100 analytes can be measured) (reviewed in Ref. 37). New computational approaches have been developed to analyze the large, high-dimensional datasets generated by CyTOF.\textsuperscript{38} The use of metal-labeled antibodies has recently been extended to the analysis of tissue sections,\textsuperscript{39,40} thus providing a highly multiplexed alternative to immunohistochemistry (IHC) in the same way that CyTOF has provided a highly multiplexed alternative to flow cytometry.

Profiles of human CD8\(^+\) T cells collected using CyTOF have shown that T cells populate a continuum of intermediary states between major states, such as naïve and memory, and that even highly specified subsets of T cells exhibit extensive combinatorial diversity in their expression of cytokines.\textsuperscript{7} These findings highlight the complexity and heterogeneity of the CD8\(^+\) T cell compartment and demonstrate the utility of CyTOF for identifying cells with transitional phenotypes.

CyTOF has also been applied to map the phenotypic diversity of the human NK cell population within and between individuals.\textsuperscript{8} Measurements of the combinatorial expression patterns of 28 NK cell receptors suggested that within a single individual, there are 6,000 – 30,000 distinct phenotypes of NK cells (out of the total \(2^{28} =\)...
268,435,456 combinations that could have been recovered by Boolean gating on the 28 measured surface markers). This analysis also found that even the 50 most common phenotypes account for an average of only 7 – 24% of the NK cells in an individual.

### 1.2.3 Single-cell secretory measurements

There is considerable interest in understanding how cytokine profiles from individual immune cells within a population correlate with outcomes of infection, vaccination, and other immunological processes. Because cytokines are important mediators of intercellular communication, single-cell secretory measurements shed light on how cells in a population interact with each other even in the absence of direct physical contact. Here, we discuss several different approaches for measuring the secretion of cytokines from single cells.

#### Approaches based on flow cytometry

ICS combined with multiparameter flow cytometry has been used to efficiently profile the production of cytokines from T cells and NK cells in a variety of experimental and clinical settings. These studies have revealed many interesting properties of cytokine secretion at the single-cell level. For example, ICS studies have shown that the breadth and magnitude of cytokines secreted by stimulated T cells shift globally over time, and that antigen-specific T cells that produce multiple cytokines ("polyfunctional" T cells) are associated with protective immune responses to pathogens such as HIV, vaccinia virus, and *Leishmania major.* Because ICS requires cells to be fixed and permeabilized prior to analysis, it cannot be used to track dynamic secretory patterns in the same individual cell over time or to recover viable, cytokine-secreting cells.

An alternative, non-destructive flow cytometric method for analyzing the secretion of cytokines from single cells is to use bispecific antibodies to link cytokines to the surface of the cell that secreted them (reviewed in Ref. 49). This method has been applied to detect and isolate antigen-specific CD8+ T cells that secreted IFN-γ or interleukin-4 (IL-4), but it is not widely used due to issues with sensitivity and
false positives. 49

Approaches based on the capture of analytes on solid surfaces

Enzyme-linked immunosorbent spot (ELISpot) assays, in which cytokines secreted by single cells are captured and subsequently detected on a membrane coated with cytokine-specific antibodies, are widely used to assess the frequencies of cytokine-secreting cells in both clinical and research settings (reviewed in Ref. 51). In addition to the detection of secreted cytokines, ELISpot assays have also been developed to measure the release of cytotoxic molecules such as perforin 52 and granzyme 53 from individual cytolytic effector cells. A dual Lysispot/ELISpot secretion-based assay has also been developed to enable the concurrent detection of target cell lysis (Lysispot) and effector cell IFN-γ secretion (ELISpot) for individual cytolytic interactions between effector and target cells. 54

ELISpot and similar methods based on fluorescent detection (Fluorospot) 55 can analyze up to three cytokines in a single assay. To increase the number of secreted analytes measured per cell, a platform known as the single-cell barcode chip (SCBC) has been developed. 56 The SCBC operates by isolating single cells in microfluidic chambers (0.54–3 nL in volume) that contain spatially barcoded arrays of capture antibodies. The cells are incubated for 12–24 h in these chambers (>1000 per device), and the cytokines they secrete are captured and subsequently detected on the spatially barcoded array. 56,57 The dense patterning of the antibody barcodes in each chamber enables detection of the secretion of 12–19 cytokines from a single cell. The SCBC has been applied to analyze the secretory profiles of macrophages, 56 tumor cells, 57 and tumor-specific patient-derived T cells. 56,58

Our lab has developed an array-based method to enable the rapid and sensitive detection of multiple secreted proteins from large numbers of single cells. In this process, known as microengraving, 59 cells are isolated in an array of subnanoliter wells (nanowells) that are each ∼125 pL. To analyze secreted cytokines (up to four per process), a glass slide coated with cytokine-specific antibodies is compressed on top of the array to capture the cytokines secreted by the cell(s) residing in each well over
a short period of time (typically one to several hours), and the resulting microarray of secreted proteins is registered back to the wells—and hence the cells—that produced the cytokines. Microengraving is non-destructive and can be repeatedly applied to measure the secretory profiles of the same individual cells at multiple points in time, thereby enabling the study of single-cell secretory dynamics and the recovery of viable cytokine-secreting cells. Furthermore, because each array contains ~80,000 to ~250,000 wells, large numbers of single cells can efficiently be interrogated. Microengraving has been applied to measure the dynamics of cytokine secretion from T cells and to recover viable T cells with defined secretory properties for downstream analysis. In the work presented in this thesis, we demonstrate that microengraving can be combined with simultaneous time-lapse microscopy (Chapter 3 and Chapter 4) and introduce strategies to increase the efficiency of microengraving when analyzing mixed populations of cells (Chapter 5).

1.2.4 Single-cell transcriptional profiling

Detailed profiles of variability in gene expression patterns are shedding light on the degree of diversity that is present among immune cells and the dynamic transitions that they undergo. Transcriptional profiling of single murine T cells using a nanofluidic platform analyzing the expression of 96 genes has shown that when different prime-boost strategies are used to deliver the same antigen, the CD8+ memory T cells that are elicited display distinct patterns of gene expression despite having equivalent frequencies in the T cell population and equivalent profiles of cytokine production. These findings illustrate how cells that share common phenotypic properties can still access a variety of unique transcriptional states dictated by their current and past experiences.

Transcriptional profiling has been extended even further by the advancement of single-cell RNA-Seq, which can be used to analyze the entire transcriptome (>10,000 genes) of single cells. Improved methods for the generation of cDNA and the preparation of sequencing libraries are making this technique increasingly feasible—from the standpoint of both cost and sensitivity—to use to profile dozens to hundreds,
and even thousands, of single cells. Applied to immune cells, single-cell RNA-Seq is proving to be a valuable tool for characterizing heterogeneity in the transcriptional response of bone-marrow-derived dendritic cells to stimulation and for the unsupervised classification of diverse types of immune cells. Furthermore, the recent development of methods to sequence RNA in situ with subcellular resolution offers the possibility of someday probing the transcriptional landscape of immune cells while also preserving information about their spatial organization in lymphoid organs, tumor tissues, or other sites of interest.

1.3 Technologies for monitoring interactions between immune cells

Many critical functions of immune cells are mediated by their contact-dependent interactions with other cells. Analyzing the dynamics of these interactions provides valuable insight into how immune cells collect and respond to signals from other cells (e.g., the activation of T cells by APCs) and how they exert their effector functions (e.g., the killing of tumor cells by NK cells). Here, we discuss how the properties of dynamic interactions between individual immune cells can be monitored in living organisms or in in vitro settings.

1.3.1 Intravital microscopy

Dynamics of T cell activation

Multiphoton microscopy is a powerful tool for analyzing dynamic interactions among individual cells in intact tissues (reviewed in Ref. 71–75), and has been used extensively to study the dynamics with which naïve T cells are primed by antigen-loaded dendritic cells (DCs) in lymph nodes. These experiments have shown that under certain conditions, T cells go through an initial phase of transient, serial interactions (typically <10 min per interaction) with DCs before progressing to a phase of long-lived (>1 h) interactions. Under other conditions, however, T cells do
not go through a prolonged phase of transient interactions but instead rapidly form stable interactions with DCs.\textsuperscript{78,79} One proposed explanation for these dynamic differences is that T cells integrate the antigenic, costimulatory, and chemotactic signals they collect from different DCs and use the cumulative signal to trigger functional responses. According to this model, a T cell may need to serially encounter many DCs presenting weak signals before reaching the same level of activation as a T cell that encounters fewer DCs that each present strong signals.\textsuperscript{80} Indeed, several experiments have suggested that T cells integrate signals from APCs over time, encounters, doses, and potencies, and then use these cumulative signals to induce outcomes such as cell division or the secretion of cytokines.\textsuperscript{79–83} These studies illustrate the importance of incorporating measurements of the dynamic properties and histories of cell-cell interactions when assessing the functional states of individual cells.

The dynamics of T cell activation have been investigated in further detail using a technique called dynamic in situ cytometry (DISC), which uses intravital multiphoton microscopy to measure the dynamics of cellular interactions while simultaneously monitoring changes in the expression of cellular surface markers.\textsuperscript{84} DISC analysis of the shedding of CD62L (indicative of signaling through the TCR) from splenic T cells has shown that T cells shed CD62L both when they form stable immunological synapses with APCs and when they form transient interactions (kinapses) with APCs, although the shedding is more rapid when stable synapses are formed.\textsuperscript{84}

Investigations of the subcellular properties of T cell-APC interfaces have provided additional details about the dynamics of T cell activation. \textit{In vivo} visualization of the dynamics of protein localization at the T cell-APC interface has been achieved by using multiphoton microscopy to monitor enhanced green fluorescent protein (EGFP) fusion proteins in T cells. This approach was used to demonstrate that downstream activities associated with antigen recognition can be induced either by transient or stable clustering of TCR molecules at the T cell-APC interface,\textsuperscript{85} and that migratory and synaptic dynamics are influenced by the history of the T cell (e.g., naïve versus recently activated) and the characteristics of the APC.\textsuperscript{86}
Dynamics of interactions between NK cells and target cells

The *in vivo* interactions between adoptively transferred murine NK cells and target cells have been characterized by two-photon intravital microscopy.\(^{87}\) NK cells residing in the lymph node migrate along collagen fibers with a mean velocity of \(6.7 \, \mu m/min\), but upon contacting target cells (allogeneic B cells) they form conjugates lasting from 1 min to >50 min and routinely display cytolytic activity.\(^{88}\) In this system, the majority of target cell interactions are monogamous. However, in some cases the contact between a target cell and an NK cell results in the recruitment of additional NK cells, suggesting that NK-to-NK communication plays a role in target cell interactions. NK cells residing in a tumor that expressed a ligand for the activating NK cell receptor NKG2D were also found to dynamically patrol the area (mean velocity \(5.2 \, \mu m/min\)) and interrogate target cells.\(^{89}\) Compared with CTLs, NK cells contacted tumor target cells for shorter periods of time, typically <10 min, but were still capable of mediating cytolysis.

1.3.2 *In vitro* microscopy for monitoring cytolytic interactions

*In vitro* video microscopy is a useful approach for precisely tracking the trajectories, contact histories, and certain functional outcomes of cell-cell interactions over long periods of time. This approach has been used by several groups to characterize the dynamics of cytolytic interactions between effector cells and their targets. For example, in the case of HIV-specific CTL clones, video microscopy studies have revealed that after killing an HIV-infected target cell, CTLs undergo a prolonged period of arrest.\(^{90}\) During this time, they remain attached to the killed target cell and fail to engage the majority of other target cells that migrate past them.

Video microscopy studies have also been used to examine how NK cells interrogate and lyse their targets. Studies of interactions between NK cells and a variety of target cells have demonstrated that the contact behavior of NK cells is dynamic, heterogeneous, and tightly regulated by the triggering of NK cell receptors.\(^{91-94}\) One
example of the heterogeneity of dynamic behaviors has come from a study of the serial killing activity of NK cells.\textsuperscript{93} This study demonstrated that over a 16 h period, human NK cells that had been expanded in IL-2 could kill up to six target cells (722.221 MHC Class I deficient cell line) in a serial manner. However, some NK cells did not kill any targets despite forming a conjugate with a target cell, illustrating the large amount of cell-to-cell variability in target cell interaction and killing ability within the NK cell population. The variable killing pattern that NK cells display when they interact with multiple target cells in series has been proposed as a metric with which to define distinct classes of NK cells.\textsuperscript{95}

One source of NK cell variability in target interaction behavior stems from the different levels and combinations of activating and inhibitory receptors expressed on NK cells. High-resolution microscopy studies have shown that activating signaling induced by contact with a target cell causes the NK cell to halt its motility and initiate the formation of a lytic synapse with the target cell, whereas inhibitory signaling limits the duration of time that the NK cell spends in contact with the target.\textsuperscript{94} The baseline activation state of the NK cell prior to encounter with a target can also significantly affect both the dynamics and outcome of the encounter. For example, NK cells that have been activated with IL-2 form longer lasting contacts with target cells and are also much more likely to mediate cytolysis compared with unactivated NK cells.\textsuperscript{96} Even NK cells that share similar dynamic properties, however, can exhibit heterogeneous killing behaviors. For example, in an experiment using IL-2-activated NK cells and 293T target cells, only 40\% of the NK cells that arrested upon contacting a target successfully lysed the target.\textsuperscript{97}

Collective interactions between groups of NK cells and target cells have been examined by \textit{in vitro} microscopy in the context of chemokine-induced NK cell migratory behavior.\textsuperscript{98} NK cells treated with monocyte chemoattractant protein-1 (MCP-1) migrated to contact K562 target cells via their leading edge while simultaneously adhering to untreated NK cells via their uropod. This tethering behavior resulted in an increased local concentration of NK cells around target cells, suggesting that chemokine-induced NK-NK adhesion may lead to cooperative behavior amongst NK
1.4 Overview of thesis

This thesis presents new sets of tools and approaches to characterize how individual immune cells interact with each other and with diseased cells.

Chapter 2 to Chaper 4 focus on the development of tools to understand how NK cells recognize, interrogate, and attack diseased target cells. The functional outcomes of interactions between NK cells and target cells are dictated by the balance between activating and inhibitory signals that NK cells receive from receptors recognizing ligands on target cells and on healthy host cells. We explore how both activating (CD16) and inhibitory (KIR and NKG2A) receptors on individual NK cells control the responses that each NK cell mounts against a target, as well as how the receptors themselves can be modulated by interactions with a target cell.

In Chapter 2, we introduce a nanowell-based platform for measuring interactions between individual NK cells and target cells. This platform enables the integrated analysis of cytolytic activity, secretory activity, and dynamic parameters of interactions between cells. Using this platform, we show that NK cells operate independently when lysing a single target cell and that lysis is most probable during an NK cell’s first encounter with a target. Furthermore, we demonstrate that the secretion of interferon-γ (IFN-γ) occurs most often among NK cells that become the least motile upon contacting a target cell but is largely independent of cytolysis.

In Chapter 3, we extend the platform to investigate how discrete contacts with individual target cells induce the shedding of CD16 (FcγRIIIA) from NK cells. NK cells use CD16 to recognize and attack antibody-coated target cells, and thus the loss of surface-expressed CD16 from NK cells is of clinical interest in the context of viral infection and antibody-based cancer therapies. We show that the loss of CD16 from an NK cell is correlated to the length of time that the NK cell spends in contact with a target cell, and that NK cells that contact antibody-coated target cells for a prolonged time almost completely shed CD16. Unlike cytolytic activity, CD16 shedding occurs...
more cooperatively than would be expected by chance. In addition, we demonstrate that although the loss of CD16 is associated with cytolytic activity and secretion of IFN-γ and CCL4, a significant fraction of NK cells that shed CD16 do not exert these common effector functions.

In addition to activating receptors such as CD16, NK cells also express a diverse range of inhibitory receptors that recognize HLA Class I molecules and prevent NK cells from attacking healthy host cells. Expression of these inhibitory receptors can confer NK cells with increased functional activity against HLA-deficient target cells in a process known as “licensing”. In Chapter 4, we apply the nanowell-based platform to study the dynamic and functional properties of individual licensed and unlicensed NK cells. We show that all NK cells secrete cytokines with a frequency that increases as they spend more time in contact with a target cell, but that unlicensed NK cells are less likely than licensed NK cells to secrete cytokines, even after prolonged contact with a target. In ongoing work, we further investigate licensing and responses to target cells by comparing the transcriptional profiles of NK cells with distinct repertoires of inhibitory receptors.

In addition to direct contact-mediated interactions, immune cells communicate with each other and with their surrounding cellular environment by secreting soluble factors such as cytokines. In Chapter 5, we develop strategies to fluorescently barcode multiple populations of immune cells and then quantify the secretory profiles of each individual cell in parallel. We apply this approach to efficiently analyze the secretory properties of immune cells treated with different doses and classes of stimuli and to compare the secretory behaviors of different lineages of CD4+ T cells.
Chapter 2

A Nanowell-Based Platform for Analyzing Interactions Between Individual Natural Killer Cells and Target Cells

This chapter is presented as it appeared in Ref. 99, with minor modifications.

2.1 Abstract

Natural killer (NK) cells are a subset of innate immune lymphocytes that interrogate potential target cells and rapidly respond by lysing them or secreting inflammatory immunomodulators. Productive interactions between NK cells and targets such as tumor cells or virally infected cells are critical for immunological control of malignancies and infections. For individual NK cells, however, the relationship between the characteristics of these cell-cell interactions, cytolysis, and secretory activity is not well understood. Here, we used arrays of subnanoliter wells (nanowells) to monitor individual NK cell-target cell interactions and quantify the resulting cytolytic and secretory responses. We show that NK cells operate independently when lysing a single target cell and that lysis is most probable during an NK cell’s first encounter with a target. Furthermore, we demonstrate that the secretion of interferon-γ (IFN-γ) occurs most often among NK cells that become the least motile upon contacting a target cell but is largely independent of cytolysis. Our findings demonstrate that integrated analysis of the cell-cell interaction parameters, cytolytic activity, and secretory activity of single NK cells can reveal new insights into how these complex functions are related within individual cells.

2.2 Introduction

Natural killer (NK) cells are granular lymphocytes classically defined by their capacity to recognize and kill tumor or virally infected cells without the need for antigen sensitization. They can also secrete cytokines and chemokines that promote the induction of a robust immune response.\textsuperscript{100,101} NK cells identify target cells using a variety of activating and inhibitory germline-encoded surface receptors.\textsuperscript{5,102} The integration of signals from these receptors regulates the cells’ cytolytic and secretory responses.\textsuperscript{12,103–106} In addition, cytokines such as interleukin-2 (IL-2) from T cells,\textsuperscript{107–110} interferon-α (IFN-α) from plasmacytoid dendritic cells (pDCs),\textsuperscript{111} and IL-12, IL-15, and IL-18 from macrophages and DCs\textsuperscript{112–116} can significantly enhance cytotoxicity.
and secretion of cytokines from NK cells.

Recent data have shown that NK cells perform several contact-dependent immunomodulatory functions in addition to directly eliminating target cells. For example, NK cells can tune the development of antiviral adaptive immunity by eliminating virus-infected DCs, by maturing and ‘editing’ pools of DCs to optimize antigen presentation, or by lysing and suppressing activated T cells. In these regulatory interactions, as well as in direct interactions with target cells, the efficacy of the NK cell-mediated immune response is determined by the cytolytic and secretory decisions that each NK cell makes upon encountering another cell. The direct relationship between cell-contact parameters, cytolysis, and the secretion of cytokines is challenging to study, however, due to the large cell-to-cell variability in both the functional capacities of NK cells and the dynamics of NK cell interactions (e.g., duration of conjugation, time to lysis, motility).

This functional diversity motivates the study of NK cell-mediated cytolysis, secretion, and interaction dynamics for individual NK cells. Conventional technologies, however, cannot efficiently match observations of these traits with single-cell resolution. Standard microscopy-based approaches offer sub-cellular imaging resolution of cell-cell interactions but are limited in throughput and are not amenable to measuring cytokine production from untransfected human cells. Lysispot/ELISpot assays enable the concurrent detection of cytolysis and secretion but are not conducive to monitoring interactions over time. Finally, flow cytometry, combined with intracellular cytokine staining (ICS), provides robust measures of the phenotypic and functional attributes of large numbers of single NK cells but decouples the history of cell-cell interactions from the functional readout.

Multiple functional properties of NK cells can be measured at the single-cell level by isolating and monitoring individual NK cells in submicroliter wells (microwells) or subnanoliter wells (nanowells). This approach has been used to demonstrate that the lysis of 293T target cells colocalized with IL-2-activated primary human NK cells in nanowells occurs with diverse dynamics—some targets display a rapid loss of calcein (a live-cell dye) when they are lysed by an NK cell, some display a slow loss of calcein, and
others remain unlysed despite being contacted by an NK cell. \cite{91} Analyses in microwells have also shown that individual primary human NK cells undergo dynamic changes in their migratory behavior by transitioning between directed migration, random migration, and periods of transient arrests in migration. \cite{97}

Microscopy-based assays using micro- and nanowells are well suited for monitoring cytolytic activity and motility, but to date there has not been an analytical process that directly integrates the measurement of these dynamic behaviors with other functional outcomes, such as secretory activity, at the single-cell level. Because one of the primary functions of NK cells is to secrete cytokines that orchestrate immune responses to pathogens and malignant cells, \cite{100,101} we developed a nanowell-based approach to concomitantly relate the secretory activity of individual NK cells to the dynamics of their interactions with target cells. Using this integrated process, we monitored hundreds of isolated NK cell-target cell interactions in parallel and analyzed the relationship between cytolytic activity, secretory activity, and motility. We found that the acute secretion of IFN-\(\gamma\) from an NK cell is associated with its motility during contact with the target cell but is not associated with the cytolytic outcome of the encounter.

### 2.3 Materials & Methods

#### 2.3.1 Cells and stimulations

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque (Sigma-Aldrich) gradient centrifugation. Study subjects were drawn from a healthy donor cohort at the Massachusetts General Hospital in Boston. The Partners Healthcare Institutional Review Board and the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects approved the study, and each subject gave written informed consent. NK cells were isolated from PBMCs by negative selection (EasySep Human NK Cell Enrichment Kit; STEMCELL Technologies) and maintained in complete media (RPMI 1640 (Mediatech) sup-
plemented with 10% heat-inactivated fetal bovine serum (PAA), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mM HEPES buffer (all from Mediatech)). Where indicated, NK cells were stimulated with IL-2 (50 U/mL; NIH AIDS Research & Reference Reagent Program), IFN-α (10 ng/mL; PBL Interferon-Source), IL-12 (10 ng/mL), IL-15 (100 ng/mL), or IL-18 (100 ng/mL) (all from R&D Systems). The major histocompatibility complex (MHC) class I-deficient K562 cell line (ATCC) was maintained in complete media and used as a model target cell.

2.3.2 Fabrication of nanowell arrays

Arrays of poly(dimethylsiloxane) (PDMS; Dow Corning) nanowells containing either 30 µm or 50 µm cubic wells were prepared by injection molding, as described previously⁹,⁶⁰,⁶² and in the Supplementary Methods (Appendix A.1.1).

2.3.3 Single-cell cytolysis assay and video microscopy

The single-cell cytolysis (SCC) assay was adapted from an assay we previously developed to identify antigen-specific cytolytic CD8⁺ T cells.⁹ The assay operates by loading small groups (≈1–5 cells/well) of effector and target cells on an array of nanowells and observing the cytolytic encounters that occur in each well (Figure 2-1). Fluorescently labeled NK cells (calcein violet, 1 µM; Invitrogen, or for video microscopy, eFluor 670, 0.5 µM; eBioscience) and target cells (CellTracker Red, 2.5 µM; Invitrogen) were adjusted to a density of 5 × 10⁵ cell/mL and loaded onto the arrays. Arrays with 30 µm or 50 µm cubic wells were used to favor the loading of single or multiple targets per well, respectively. Media containing SYTOX green (nucleic acid stain to identify dead cells, 0.5 µM; Invitrogen) was applied and the arrays were covered with lifter slips (Electron Microscopy Sciences) or glass slides.

Arrays were imaged using an automated inverted epifluorescence microscope (Axio Observer; Carl Zeiss, 10×/0.3 objective) fitted with an EM-CCD camera (ImagEM; Hamamatsu). For the SCC assay, images were collected at 0 h to determine the initial occupancy of each well and at 4 h to validate the occupancy and determine
the acquisition of SYTOX by targets. Arrays were incubated at 37°C and 5% CO₂ between the initial and final images. Video microscopy was performed in a similar manner by imaging a subsection of the array at 8 min intervals over a total of 4 h. A custom-written script was used to process the images.

2.3.4 Microengraving to detect secreted proteins

The secretion of cytokines and chemokines from the cells residing in each nanowell was measured by microengraving using previously reported protocols. Microengraving measures secretory events from both single- and multi-celled wells, but it does not directly attribute secretions from multi-celled wells to specific cells within the well. (The cells’ respective contributions may be deconvolved based on independently determined knowledge of cellular properties (e.g., certain cells do not secrete specific analytes under certain conditions).) Secreted macrophage inflammatory protein-1β (MIP-1β, or CC chemokine ligand 4 (CCL4)) and IFN-γ from each well were captured for 1 h (30 µm wells) or 2 h (50 µm wells) on a glass slide coated with the corresponding capture antibodies (Figure 2-6). The resulting microarray of captured proteins was then probed with fluorescent detection antibodies and imaged. See Supplementary Methods (Appendix A.1.2) for additional details.

2.3.5 Functional and phenotypic flow cytometry

For the functional flow cytometric assays, 10⁶ PBMCs were pre-incubated in the presence or absence of stimulatory cytokines (as indicated in the text), and then incubated with K562 target cells at an effector:target (PBMC:K562) ratio of 10:1 in the presence of 0.5 µg/mL Brefeldin A (Sigma-Aldrich), 0.3 µg/mL Monensin (Golgi-Stop, BD Biosciences), and anti-CD107a-PE-Cy5 antibody (BD Biosciences). After 4 h of incubation, cells were stained using the LIVE/DEAD Fixable Blue Dead Cell Stain (Invitrogen), washed, and then incubated for 15 min with the following antibodies: anti-CD3-Pacific Blue (PB), anti-CD14-PB, anti-CD19-PB, anti-CD16-allophycocyanin (APC)-Cy7, and anti-CD56-phycoerythrin (PE)-Cy7 (all BD Bio-
Cells were then fixed and permeabilized using the Cytofix/Cytoperm solution kit (BD Biosciences) according to the manufacturer’s instructions. Intracellular cytokine staining was performed for 30 min with anti-IFN-γ-fluorescein isothiocyanate (FITC) and anti-MIP-1β-PE (both BD Biosciences).

To assess how the different stimulation conditions might affect the phenotypes and activation states of NK cells, PBMCs were separately stained with the immunophenotypic markers anti-NKG2D-APC, anti-NKp46-PE (both BD Biosciences), anti-CD69-FITC, and anti-perforin-peridinin chlorophyll protein (PerCP)-Cy5.5 (both eBioscience). In all experiments, NK cells were defined as live CD3–CD14–CD19– lymphocytes expressing CD56 and/or CD16 (Figure A-1). In separate experiments, surface expression of NKG2D ligands on K562 cells was confirmed using anti-UL16 binding protein (ULBP)-1-PE, anti-ULBP-2-PE, anti-ULBP-3 (primary) (all from R&D Systems) with goat anti-mouse-APC (secondary), and MHC class I chain-related protein A/B (MICA/B)-PE (both BD Biosciences) (Figure A-2). Data were acquired on a BD LSRII (BD Biosciences) flow cytometer and analyzed using FlowJo software (V9.3.1; Tree Star).

2.3.6 Data analysis

Raw data were processed as described above and in the Supplementary Methods (Appendix A.1.2). In all experiments, the wells that were located on the edge of the array were excluded from analysis to minimize any potential edge effects. To analyze the video microscopy data, a custom-written MATLAB (R2010b; MathWorks) script was used to calculate parameters describing the interactions of single NK cells with up to four target cells. Intervals of NK cell-target cell contact were manually verified. Custom-written MATLAB scripts were used to correlate data from the SCC assay or video microscopy with data from microengraving on a per-well basis. Statistical analyses were performed with Prism 5 (GraphPad Software); specific statistical tests are indicated in the text.
2.4 Results

2.4.1 Individual NK cells lyse target cells when co-incubated in nanowells

We developed a single-cell cytolysis (SCC) assay to directly measure the cytolytic behavior of thousands of individual NK cells (Figure 2-1). NK cells and K562 target cells were co-deposited onto an array of 30 $\mu$m cubic nanowells to obtain small, isolated groups of cells. The stochastic loading procedure resulted in the majority of the nanowells being filled with 0–3 cells of each type (Figure A-3). For the SCC assay, analysis was restricted to the 1,000–5,000 wells per array that contained exactly one NK cell and one target cell. After 4 h of co-incubation in the nanowells, productive cytolytic interactions were identified by the appearance of SYTOX$^{+}$ target cells (Figures 2-1, 2-2A). These initial experiments established that thousands of cytolytic and non-cytolytic interactions between individual NK cells and targets could be observed in parallel.

The cytolytic potential of NK cells is strongly modulated by cytokines that are released locally during an immune response.$^{108-111,114-116}$ We therefore assessed how stimulation with exogenous cytokines affected the cytolysis measured in the SCC assay (Figure 2-2B). Prior to the assay, NK cells were incubated for 20 h with media, IL-2, IFN-α plus IL-2, or a combination of IL-12, IL-15, and IL-18. In the absence of cytokine stimulation, 7% ± 6% of NK cells in nanowells containing a single NK cell and a single target lysed the target. In each experiment, this rate was significantly above the 2% ± 1% background death rate of single target cells on the same array ($P < .0001$, Chi-squared with Yate’s correction). Activation of NK cells with exogenous cytokines prior to the assay resulted in a significant increase in the frequency of target cell death (23–26%) compared with unstimulated NK cells ($P < .05$, one-way analysis of variance (ANOVA) followed by Tukey’s post-test) (Figure 2-2B).

The cytolytic potential of NK cells$^{11}$ and T cells$^{10}$ is associated with target cell-induced surface expression of CD107a, a degranulation marker. Assays employing
Figure 2-1: Schematic of the single-cell cytolysis (SCC) assay. NK cells and K562 target cells are loaded onto an array of nanowells. An initial set of images is acquired to determine the number of cells per well; after 4 h of incubation, a second set of images is acquired to identify wells in which a killing event occurred (SYTOX+ target cell).

Flow cytometry commonly use CD107a as a surrogate measurement for the cytolytic activity of single NK cells. We therefore compared the frequency of single-cell cytolysis (measured by the SCC assay) with the frequency of NK cells expressing CD107a after 4 h of co-incubation with targets (measured by flow cytometry) (Figure 2-2B). For each stimulation condition, the frequency of NK cells that upregulated CD107a expression was higher than the frequency of cytolytic NK cells observed in the SCC assay, but the two measurements were significantly correlated ($R^2 = 0.89, P < .05$, Pearson correlation). The discrepancy in the absolute frequencies may be explained by differences in bulk compared to single-cell stimulation, by the fact that CD107a can be expressed on the surface of NK cells that have been activated but have not lysed a target, or by the fact that the acquisition of SYTOX signal marks the end-stages of target cell death (membrane permeabilization). Overall, these results demonstrated that the SCC assay monitors the cytolytic outcome of thousands of individual NK cell-target cell pairs, and that the cytolytic frequencies measured in this assay reflect the cytolytic activity as measured in bulk cultures.
Figure 2-2: NK cells interrogate and lyse K562 target cells when co-incubated in nanowells. (A) Representative images of NK cells (blue) co-incubated with target cells (red) in the nanowells. Lysed target cells become positive for SYTOX (green). (B) Frequencies of cytolytic events measured by the SCC assay and of degranulated NK cells measured by flow cytometry after 4 h of NK cell-target cell interactions. NK cells were incubated for 20 h with the indicated stimulation conditions prior to either assay. Data from the SCC assay were gated to include wells containing a single NK cell and a single target. Target cell-induced degranulation is expressed as ∆CD107a, which indicates the difference in expression of CD107a on the surface of NK cells that were or were not exposed to target cells in bulk. Mean and standard deviation (SD) for at least three donors per condition are shown. ###P < .0001, Chi-squared with Yate’s correction comparison of lysis rates of target cells in wells on the same array containing zero NK cells (background death) or one NK cell. *P < .05, **P < .01, ***P < .001, one-way ANOVA followed by Tukey’s post-test.
2.4.2 Small groups of NK cells do not cooperate to kill single target cells

We next quantified the frequency of cytolysis in nanowells containing different numbers of NK cells (zero, one, two, or three NK cells) in the presence of a single target to determine whether NK cells kill more efficiently in groups than in isolation (Figure 2-3A). As expected, increasing the number of NK cells in the well led to increased frequencies of cytolysis. To determine whether this increase occurred in a cooperative or independent manner, we tested if the observed frequency of lysis in wells containing one NK cell could predict the frequency of lysis in wells containing multiple NK cells. If NK cells act independently when killing a single target, then the predicted frequency of dead targets in wells with \( n \) NK cells is

\[
1 - (1 - P_{\text{death}})^n
\]  

(2.1)

where \( P_{\text{death}} \) is the frequency (probability) of the target cell being lysed when co-located with a single NK cell. We found that the observed frequencies of lysis in wells with multiple NK cells were not significantly different from the predicted frequencies (\( P > .05 \) for 23 of the 24 cases tested, Fisher’s exact test) (Figure 2-3B). This result suggested that NK cells residing in small groups in nanowells operate independently to mediate the acute lysis of a single target cell; in other words, they do not attack more effectively as a group than as individuals (e.g., by enhancing the cytolytic behavior of neighboring NK cells or by delivering partial lytic hits that cumulatively, but not individually, result in lysis). We note that cooperative effects over larger length scales \textit{in vivo} could occur by the selective recruitment of specific subsets of NK cells to the site of infection or tumorigenesis.
Figure 2-3: **NK cells act independently when lysing a single target cell in a nanowell.** (A) Representative distribution of SYTOX intensity in target cells after being co-incubated for 4 h in nanowells with one, two, or three NK cells. Data were gated to include wells containing a single target cell. Example images show the initial occupancy of the nanowells for each combination. The percentage of lysed target cells (double-positive for SYTOX and CellTracker Red) is indicated. In this example, the NK cells were obtained from the donor depicted in gold in the plots in Panel B and were activated with IFN-α plus IL-2. MFI, median fluorescence intensity. (B) Observed frequencies of cytolysis (filled circles; colors indicate unique donors) in nanowells containing 0–3 NK cells. For each donor, the frequencies of cytolysis predicted from the frequency observed in wells containing a single NK cell are shown as dotted lines. Prior to the assay, NK cells were stimulated with the indicated conditions for 20 h.
2.4.3 IL-2-activated NK cells lyse target cells with heterogeneous dynamics and in an order-dependent manner

The induction of functional responses in NK cells depends heavily on the dynamics of contact between NK cells and the target cells that they interrogate.\textsuperscript{94,125} To characterize the dynamics of contact, we performed time-lapse microscopy on a subsection of the array to collect movies of single NK cells interrogating 0–4 target cells in 50 \( \mu \text{m} \) cubic wells (Figure 2-4). NK cells were stimulated for 44 h in 50 U/mL IL-2 prior to the assay to ensure a robust functional response. This stimulation activated NK cells (47% ± 9% CD69\textsuperscript{+}) but did not change the expression of NKp46, NKG2D, or perforin compared with unstimulated NK cells (Figure A-4).

NK cells patrolled the nanowells and formed multiple contacts with targets in the wells (Figure 2-5A). NK cells that formed contacts with targets mediated the cytolysis of one or more targets in 25% of the cases (81 out of 324, compiled from two donors). Among cytolytic NK cells, the duration of contact with a target prior to lysis (marked by membrane permeabilization) ranged from less than 8 min to over 200 min (Figure 2-5B). This wide distribution in times implies that the initiation and execution of cytolytic activity following contact with a target occurs with heterogeneous dynamics.

The probability of a target cell being lysed depended upon the order in which it was contacted by the NK cell—the first target that an NK cell encountered was more likely to be lysed than targets that were encountered subsequently (Figure 2-5C). A decrease in cytolytic activity over sequential encounters with target cells could occur if the cytolytic potential of an NK cell is diminished after the delivery of a lytic hit. To test this hypothesis, we examined whether NK cells that lysed the first target they encountered were able to lyse other target cells upon subsequent contact. Among the NK cells that contacted 2–4 target cells, 27% (37 out of 135) lysed the first target that they encountered. Within the group of cells that exhibited cytolytic activity upon the first encounter, 19% (7 out of 37) also lysed additional targets that they encountered later. This result suggested that NK cells retain their cytolytic potential after delivering a lytic hit (consistent with previous reports of serial killing\textsuperscript{93} and

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bulk studies demonstrating that NK cells retain perforin and granzyme loading after target cell-induced degranulation\textsuperscript{127}), but that only a subset of the initially cytolytic NK cells proceed to serially lyse multiple targets. In addition, among the group of NK cells that did not lyse the first target, 93\% (92 out of 98) also did not lyse any of the other targets that they subsequently encountered. This result demonstrated that NK cells that do not lyse the first target they encounter are unlikely to exhibit cytolytic activity upon subsequent encounters with similar target cells within short periods (4 h). Together, these results suggest that the decrease in cytolytic activity over sequential encounters with target cells occurs because (1) only a fraction of NK cells that lyse the first target they encounter continue on to lyse additional targets, and (2) NK cells that do not lyse the first target they encounter are unlikely to lyse targets that they encounter later.

2.4.4 Microengraving measures the short-term secretory activity of individual NK cells

In addition to their cytolytic activity, NK cells also contribute to immune control by secreting cytokines and chemokines. We used microengraving\textsuperscript{9,59–62,126} a technique to detect the proteins that are secreted by the cells residing within each nanowell on the array (Figure 2-6), to measure the short-term secretory profiles of individual NK cells and match them to the corresponding observations of the NK cell-target cell interactions (Figure 2-7A). We focused our analysis on the secretion of MIP-1\(\beta\) and IFN-\(\gamma\). These proteins are secreted by NK cells and play important roles in coordinating the immune response. MIP-1\(\beta\) is a chemokine that can attract NK cells\textsuperscript{128} and other immune cells; it also suppresses human immunodeficiency virus (HIV) infection by inhibiting the binding of HIV to CC chemokine receptor type 5 (CCR5).\textsuperscript{19,20} IFN-\(\gamma\) is a cytokine that inhibits viral replication, increases the presentation of antigen, and polarizes the differentiation of T helper cells to the Th1 lineage.\textsuperscript{129}

We first validated that microengraving could detect characteristic patterns of secretory activity from NK cells that interacted with target cells. Prior to microen-
Figure 2-4: **Automated tracking of interactions between NK cells and target cells.** Representative composite micrographs of a single IL-2-activated (50 U/mL, 44 h) NK cell (blue) interacting with a target cell (red) and inducing cytolysis (green; SYTOX). Images were acquired every 8 min for 4 h. For each target cell, the SYTOX intensity and the distance to the NK cell were calculated using automated tracking scripts.
Figure 2-5: **Dynamics of cytolytic interactions between NK cells and target cells.** (A) Distribution of the number of contacts formed by single NK cells with distinct target cells over 4 h as a function of the total number of target cells residing in each nanowell. (B) Histogram of the total NK cell-target cell contact time required for cytolytic NK cells to induce the permeabilization of the membrane of the target cell. Data are segregated into pairs of NK cells and target cells that were not yet in contact in the first image (dark grey bars) or were already in contact in the first image (light grey bars). (C) Relative probability of an NK cell lysing the first, second, third, or fourth target cell that it encountered. Groups are segregated based on the total number of distinct target cells that were encountered, and relative probabilities were calculated as the fraction of lysed target cells in each group that were the $n^{th}$ target cell to be contacted by the NK cell. Panels A and B are representative of two independent donors; Panel C shows combined data from both donors.
graving, NK cells were incubated for 20 h in either media alone or a combination of IL-12, IL-15, and IL-18. NK cells were then loaded with target cells into 30 µm cubic nanowells and incubated for 4 h to allow interactions with targets to take place. Following incubation, microengraving was performed for 1 h to capture secreted MIP-1β and IFN-γ, and wells that contained a single NK cell and multiple target cells were analyzed for secretion (Figure 2-7B). Previous reports have shown that, in the absence of activation with exogenous cytokines, MIP-1β is the dominant secretory product from NK cells that interact with K562 target cells. Similarly, we found that MIP-1β was secreted by a small but consistently detectable fraction of NK cells that were cultured in media alone prior to being introduced to the target cells in the nanowells. NK cells that were activated with IL-12, IL-15, and IL-18 prior to the assay exhibited increased secretion of MIP-1β and strong secretion of IFN-γ. This finding was consistent with previous reports showing that IL-12, IL-15, and IL-18 activate NK cells and induce the secretion of cytokines, especially IFN-γ. Analogous assays performed using ICS in combination with flow cytometry produced similar trends in relative secretory activity (Figure 2-7B). Together, these experiments confirmed that microengraving can be used to profile the secretion of MIP-1β and IFN-γ from NK cells interacting with target cells in the array of nanowells.

2.4.5 Secretion of IFN-γ from IL-2-activated NK cells is associated with reduced motility during contact with target cells

As a population, NK cells patrol their local microenvironment (e.g., lymph node or tissue), interrogate target cells, lyse target cells, and secrete cytokines. How these diverse functional parameters are related in each individual NK cell, however, has not been well characterized. To address this question, we matched observations of interactions (determined by time-lapse microscopy) between single IL-2-activated (50 U/mL, 44 h) NK cells and target cells in 50 µm cubic wells to measurements of secreted MIP-1β and IFN-γ collected via microengraving from the same nanowells (Figure 2-
Figure 2-6: Schematic of microengraving to detect secreted cytokines and chemokines. A capture antibody-coated glass slide is placed in contact and incubated with the array of nanowells; this process creates a matched microarray of secreted proteins that is subsequently detected with fluorescently labeled antibodies, imaged, and analyzed to correlate secretion events to the nanowell of origin.
Figure 2-7: Microengraving measures the short-term secretory activity of individual NK cells. (A) Representative matched images measuring cytolytic activity and the secretion of proteins from the same NK cell. Cells are labeled as described in Figure 2-2A. (B) Production of cytokines and chemokines measured by microengraving or intracellular cytokine staining (ICS). NK cells were incubated for 20 h with the indicated stimulation conditions prior to either assay and then incubated with target cells in the nanowells (microengraving assay) or in bulk (ICS assay) for 4 h. The microengraving data were gated to include wells containing a single NK cell and multiple target cells. Mean and SD for at least three donors per condition are shown. Note difference in scale between the two graphs.
The resulting collection of functional profiles allowed us to quantitatively analyze cytolysis, secretion, and the dynamics of interaction in individual NK cells.

We first examined whether target cell-induced secretory activity and cytolytic activity were associated for individual NK cells. NK cells that contacted at least one target cell displayed robust secretion of MIP-1β and IFN-γ (Figure 2-9A). Secretion was not observed in the absence of contact with a target, even if a target cell was present in the well. Among NK cells that contacted at least one target, however, there was no significant difference in the secretion of MIP-1β or IFN-γ between the cytolytic and non-cytolytic groups of NK cells (Figure 2-9A). These results suggest that acute, target cell-induced secretory activity is not closely correlated to cytolytic activity in individual IL-2-activated NK cells.

To investigate how the dynamics of contact with target cells may regulate cytolysis and secretion in single NK cells, we next examined whether the motility of NK cells during contact with targets was associated with the subsequent cytolytic or secretory outcome of the encounter. The velocities of NK cells during periods of contact with target cells were reduced compared with the velocities in the absence of targets or after disengagement from a target (Figure 2-9B; \( P < .001 \), Kruskal-Wallis test followed by Dunn’s post-test). When the velocities of NK cells during contact with targets were further segregated based on the functional response (MIP-1β+/+, IFN-γ−/+, lysis−/+), we found that cells that secreted IFN-γ had a significantly reduced velocity during contact with targets compared with cells that did not secrete IFN-γ (Figure 2-9C; \( P < .05 \), Mann-Whitney test), and that these cells remained more arrested at the initial point of contact with the target (Figure 2-10). There was no significant difference between the during-contact velocities of NK cells that were segregated by the secretion of MIP-1β or cytolytic outcome (Figure 2-9C). Together, these results suggest that NK cells receive stop signals of varying strengths after contacting a target, and that the degree of arrest in motility is related to the subsequent secretion of IFN-γ.
Figure 2-8: Correlated measurement of the dynamics of NK cell-target cell interactions and secreted products. Representative composite micrographs of IL-2-activated (50 U/ml, 44 h) NK cell-target cell interactions and corresponding measurements of secreted chemokines and cytokines. Interactions were tracked for 4 h at 8 min intervals using time-lapse microscopy (labeling as described in Figure 2-4); microengraving was performed immediately afterward for 2 h to capture chemokines and cytokines secreted by each NK cell.
Figure 2-9: Secretion of IFN-γ is associated with the velocity of NK cells during contact with target cells. (A) Secretion of MIP-1β and IFN-γ from single NK cells. NK cells are segregated based on whether they contacted a target cell, and, if they did contact at least one target cell, whether they exhibited cytolytic activity. Dashed lines indicate the threshold for positive secretion events (background + 2 SD). **P < .01, ***P < .001, n.s., not significant, Mann-Whitney test. (B) Mean velocities of NK cells in nanowells in the absence of target cells (0 K562), during contact with target cells, and after contact with target cells. ***P < .001, Kruskal-Wallis test followed by Dunn’s post-test. (C) Mean velocities of NK cells in nanowells during contact with target cells. Data are segregated by functional outcome. *P < .05, n.s., not significant, Mann-Whitney test. The mean and standard error of the mean (SEM) are indicated in red. Results are representative of two independent donors.
Figure 2-10: **Cell tracks segregated by functional outcome.** Tracks of NK cells in the absence of target cells (left) or during contact with a target cell (right). The tracks during contact are segregated by secretory outcome. Each plot displays $n = 15$ tracks that were randomly selected from the pool of all eligible cell tracks. Results are representative of two independent donors.

### 2.5 Discussion

NK cells induce cytolysis and secrete chemokines and cytokines in response to malignant or infected cells, and thus play a critical role in the immune control of cancer and infectious diseases.$^{101,130}$ Characterizing how individual NK cells interact with, and subsequently respond to, potential target cells is important for understanding the mechanisms by which they eliminate targets but leave healthy cells unharmed. Our group has previously described the use of arrays of nanowells to screen for HIV-specific CD8$^+$ T cells.$^9$ Here, we extended this approach to monitor interactions between individual NK cells and K562 target cells and then correlated these interactions to linked measurements of the cytolytic and secretory outcomes from the individual NK cells. The results of these dynamic, single-cell measurements begin to define the rules of engagement that modulate the functionality of NK cells during their response to target cells.

The unique ability of the SCC assay to precisely count the number of NK cells and targets in each nanowell allowed us to quantitatively investigate whether small
groups of NK cells act cooperatively when killing an isolated target cell. Our results were consistent with a model in which each NK cell operates as a cytolytic agent that is independent of other NK cells present in the well. The lack of cooperative cytolytic behavior suggests that local paracrine signaling (over the sub-nanoliter volume of the well) between NK cells does not induce them to work synergistically to kill single target cells within a 4 h interval. This finding, however, does not eliminate the possibility for cooperative killing behavior among NK cells when killing multiple targets, killing resistant targets, or operating over larger length scales or longer intervals of time. Indeed, the bulk self-recruitment of NK cells into regions containing a high density of target cells is commonly observed. In contrast, the self-recruitment of individual NK cells to aid in the acute elimination of a single target cell has been observed only on occasion, thus supporting our finding that local cooperative behavior among NK cells is not a major mechanism by which the lysis of single, susceptible targets is achieved.

The variability in time to lysis that we observed may involve mechanisms by which NK cells progress through cytoskeletal polarization checkpoints before delivering a successful lytic hit or cell-to-cell differences in the kinetics of apoptosis in targets. NK cells lyse K562 target cells primarily by delivering perforin/granzyme-loaded cytolytic granules into the lytic synapse rather than by triggering death receptor-mediated pathways of apoptosis induced by Fas ligand (FasL) or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Some of the heterogeneity in lysis times and behaviors, however, could result from NK cells inducing the death of target cells by other mechanisms—for example, by using membrane nanotubes to facilitate cytolytic interactions with target cells.

Our finding that cytolytic NK cells are most likely to lyse the first target cell they encounter suggests that they do not need to integrate activating signals collected during prior encounters with targets before overcoming the signaling threshold required for the delivery of a lytic hit; instead, they exert their cytolytic activity at the first opportunity. This pattern of activation is different from the pattern followed by T cells during priming in the lymph node under certain conditions, where it has been
shown that the signals received through the T cell receptor are integrated over serial encounters with antigen-presenting cells until a threshold of activation is reached. Because both the density and the type of ligands displayed by target cells have a large effect on the functional response of NK cells, it will be interesting to test whether different target cells produce different patterns of order-dependent cytolytic activity.

Cytolysis and secretion are the primary functions of NK cells during an immune response. By collecting linked measurements of the cytolytic and secretory activities of individual IL-2-activated NK cells upon exposure to target cells, we demonstrated that the acute secretion of IFN-γ and MIP-1β is not associated with the cytolytic status of NK cells. Studies using bulk mixtures of NK cells and target cells have also suggested that cytolysis and secretion may be differentially regulated or temporally distinct. Furthermore, cytokines (e.g., IFN-γ, TNF) and perforin-loaded cytolytic granules are sorted into separate endosomes and released in distinct fashions from NK cells, providing mechanistic support to the idea that cytolysis and secretion can be controlled independently within individual cells.

The relationship between the motility of NK cells and their secretory responses to target cells has not previously been analyzed at the single-cell level. Using integrated measurements of single, IL-2-activated NK cells, we found that target cell-induced secretion of IFN-γ was associated with the motility of the NK cells during contact with targets. The during-contact velocity of NK cells that secreted IFN-γ was significantly lower than that of NK cells that did not secrete IFN-γ. Previous reports have demonstrated that NK cells display a dose-dependent reduction in velocity when exposed to increasing concentrations of surface-bound MICA, an NKG2D ligand expressed on K562 target cells, and that these activating signals induce NK cells to arrest and form stable synapses with target cells. It has also been shown that the level of activation that is necessary to induce the secretion of IFN-γ is higher than that necessary to induce degranulation or the secretion of MIP-1β. Our findings, therefore, are consistent with a model in which NK cells that receive the strongest activating signals upon encounter with a target have the lowest during-contact velocity (due to
receiving a strong stop signal) and are also the most likely to surpass the signaling threshold required to induce the secretion of IFN-γ. A similar association between antigen-induced arrest and the secretion of IFN-γ has also been observed at the population level in mouse CD4+ T cells.\textsuperscript{143} Interestingly, we did not detect a significant difference in the during-contact motility of cells that did or did not secrete MIP-1β (a chemokine), although in other systems of interacting lymphocytes the secretion of chemokines has been shown to affect the characteristics of the immune synapse.\textsuperscript{144} In general, the association between the during-contact velocity and the secretion of IFN-γ suggests that, for NK cells, there is overlap in the target cell-triggered signaling pathways that control motility and those that control the secretion of cytokines.

In summary, we have developed and validated a nanowell-based platform to quantitatively monitor and correlate the contact dynamics and functional outcomes (cytolysis, secretion) of interactions between individual NK cells and target cells. Integrated measurements such as these have not previously been possible to perform at the single-cell level due to the constraints of conventional assays. In the present study, we analyzed interactions between NK cells and tumor target cells, but the nanowell-based platform presented here could also be used to examine cross-regulation between NK cells and virally infected cells, DCs, or T cells with single-cell resolution. Moreover, the platform is suitable for use with samples containing a limited number of cells ($10^4$–$10^5$), and thus will enable unprecedented functional analysis of clinical biopsy specimens. Such applications could include the analysis of mucosal NK cells in HIV infection\textsuperscript{145} or of intrahepatic NK cells in viral hepatitis.\textsuperscript{146} In the current study, which focused on interactions between peripheral blood NK cells and K562 tumor target cells, we demonstrated that local cooperativity among NK cells does not contribute significantly to the lysis of single target cells, and that short-term secretory responses arising from individual NK cells are correlated with the dynamics of NK cell-target cell interactions but not cytolysis. Together, these results illustrate new properties by which NK cells perform immune surveillance and mediate the elimination of target cells.
Chapter 3

Dynamic and Functional Correlates of Activation-Induced Shedding of CD16 from Natural Killer Cells after Discrete Interactions with Target Cells
3.1 Abstract

FcγRIIIA (CD16) is an activating receptor that is expressed on ~90% of resting natural killer (NK) cells. CD16 mediates the recognition and cytolysis of antibody-coated target cells by NK cells, but it is proteolytically shed upon stimulation by target cells. This shedding renders NK cells deficient in responding to subsequently encountered antibody-coated target cells. The dynamics by which and extent to which CD16 is shed from an NK cell during a discrete interaction with a single target cell are unknown. Here, we addressed this question by using video microscopy to track the dynamic interactions between individual NK cells and target cells and then measuring the amount of CD16 that remained on the surface of the NK cells at the conclusion of the interaction. By profiling thousands of pairs of interacting NK cells and target cells with a distribution of contact times, we found that the loss of CD16 from an NK cell was directly correlated to the length of time that the NK cell spent in contact with a target cell. Contact with a single antibody-coated target cell was sufficient to induce the loss of CD16. The length of contact time required to induce the loss of CD16 was shorter in IL-2-activated NK cells than in resting NK cells. Loss of CD16 was associated with the performance of effector functions such as lysis of the target cell and secretion of cytokines and chemokines, but a significant fraction of NK cells that shed CD16 did not exert any of the effector functions that we assessed (killing, secretion of IFN-γ, secretion of CCL4). Furthermore, confined groups of two or three NK cells (with at least one target cell) were more likely to all shed CD16 than would be expected by chance, which suggests that shedding occurs in a cooperative fashion. Together, these results demonstrate that prolonged engagement of a single antibody-coated target cell is sufficient to induce the shedding of CD16 but that NK cells that shed CD16 do not necessarily perform effector functions such as cytolysis or the secretion of IFN-γ or CCL4.
3.2 Introduction

Antibodies that recognize antigens on diseased target cells can be generated naturally by humoral immune responses (e.g., non-neutralizing antibodies against viral pathogens) or administered as part of treatment regimes (e.g., tumor-targeting monoclonal antibodies). The Fab (Fragment antigen binding) regions of these antibodies bind to the antigens expressed on the target cell, while the Fc (Fragment crystallizable) regions are left exposed on the surface of the cell. Innate immune cells, including natural killer (NK) cells, macrophages, monocytes, and granulocytes, are equipped with receptors that recognize the Fc regions of antibodies and induce effector mechanisms that lead to the destruction of antibody-coated target cells.\textsuperscript{147}

The majority (\textasciitilde 90\%) of CD56\textsuperscript{dim} NK cells express the receptor Fc\(\gamma\)RIIIA (CD16), which binds with low affinity to the Fc region of certain subclasses of immunoglobulin G (IgG).\textsuperscript{100,147–149} Upon encounter with an antibody-coated target cell, the CD16 receptors on NK cells bind to the Fc regions of the coating antibodies. The resulting engagement of CD16 induces the phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptor molecules including CD3\(\zeta\) and Fc\(\gamma\)RI\(\gamma\), which trigger a cascade of activating signaling.\textsuperscript{106,150,151} These activating signals can stimulate NK cells to mediate antibody-dependent cellular cytotoxicity (ADCC) by directing cytolytic granules into the target cells. In addition to killing target cells via ADCC, stimulation through CD16 also induces NK cells to secrete anti-viral and immunostimulatory cytokines and chemokines such as interferon-\(\gamma\) (IFN-\(\gamma\)), macrophage inflammatory protein-1\(\beta\) (MIP-1\(\beta\), or CC chemokine ligand 4 (CCL4)), and tumor necrosis factor-\(\alpha\) (TNF).\textsuperscript{12} Unlike other activating receptors on NK cells, which only induce robust cytolytic or secretory responses when they are synergistically triggered in combination, engagement of CD16 alone is sufficient to induce strong effector responses from NK cells.\textsuperscript{103,104}

The activation of NK cells by a variety of stimuli results in the loss of CD16 from the cell surface. Expression of CD16 is significantly reduced on NK cells that have been treated with phorbol 12-myristate 13-acetate (PMA),\textsuperscript{152–154} which potently
stimulates immune cells by binding and activating protein kinase C (PKC). Surface-expressed CD16 is also reduced on NK cells after exposure to activating cytokines such as interleukin-2 (IL-2), IL-12, IL-15, and IL-18.\textsuperscript{153,154} The engagement of activating receptors on NK cells (CD16, NKG2D, etc.), either by antibody-coated plates or by target cells, also leads to loss of CD16.\textsuperscript{153,155–157} Interestingly, one of the most potent inducers of the loss of CD16 is the engagement of and stimulation through CD16 itself.\textsuperscript{153}

Receptors such as CD16 can be removed from the surface of cells by internalization or shedding. Although the application of soluble anti-CD16 to NK cells induces the internalization of CD16,\textsuperscript{158} several lines of evidence suggest that metalloprotease-induced shedding drives the loss of CD16 following other routes of activation. After activation with PMA or IL-12 and IL-18, soluble CD16 fragments can be detected in the supernatant, suggesting that CD16 is cleaved from the surface of activated NK cells rather than (or perhaps in addition to) being internalized.\textsuperscript{152,153} Furthermore, stimulation of NK cells in the presence of phenylarsine oxide (PAO), which disrupts endocytosis, does not stop the loss of CD16 from the surface of the cells.\textsuperscript{154} In contrast, retention of surface-expressed CD16 on activated NK cells is significantly increased when the cells are treated with broad-spectrum inhibitors of proteases or more selective inhibitors of specific matrix metalloproteinases (MMPs) or proteases from the ADAM (a disintegrin and metalloproteinase) family.\textsuperscript{152–157} In support of these findings, ADAM17 and MMP25 have recently been shown to cleave CD16 from the surface of activated NK cells.\textsuperscript{153,154,157}

The shedding of CD16 from the surface of NK cells after encounters with target cells is of clinical interest, because the loss of this receptor renders NK cells deficient in their ability to mediate ADCC or to mount other effector functions in response to antibody-coated target cells.\textsuperscript{156} In many disease settings, target cells are a minority population amidst a sea of healthy cells (e.g., antibody-coated HIV-infected CD4\textsuperscript{+} T cells in the blood, or malignant NKG2D\textsuperscript{+} cells in precancerous tissue). Experimentally, however, it is difficult to quantify on a per-cell basis the relationship between the stimulation that an NK cell receives from individual encounters with target cell(s)
and the loss of CD16 (or other functional outcomes of interest). This difficulty stems from the technical challenge of correlating the effective “dose” of target cells that any given NK cell is exposed to with the subsequent shedding of CD16 from the NK cell.

Standard experimental protocols commonly modulate the global amount or type of stimulation that is presented by the population of target cells. Global modulation can be achieved by varying the coating density of activating ligand (e.g., IgG) on the surface of the target cells or by using target cells that stimulate through different activation receptors on NK cells. Although this type of modulation provides useful information about stimulatory outcomes, it does not enable control over the local concentration of target cells that each NK cell is exposed to. For example, in a bulk mixture, an NK cell may have the opportunity to interact with tens or hundreds of target cells over the course of the assay.

One approach to limit the number of target cells encountered by an NK cell is to use vastly more non-target cells than target cells in the cellular mixture. Although this approach would ensure that the majority of NK cells would encounter at most a single target, it would not answer the question of whether an NK cell that retains CD16 on its surface does so because it did not encounter a target cell (and hence was not stimulated) or because it encountered a target cell but did not shed CD16. Target-induced stimulation can also be controlled by restricting the co-incubation time; however, this approach precludes the analysis of responses that are induced by an interaction with a single target cell but that require time to develop. Instead, it would be desirable to measure the phenotypic and functional responses (such as the shedding of CD16) of NK cells in a way that links each cell’s response to the duration of stimulation that it received from a discrete encounter with a target cell.

Here, we extend the nanowell-based platform introduced in Chapter 2 to enable measurement of the shedding of CD16 from NK cells that engaged a single or small number of target cells for varying durations of time. We also correlate the activation-induced shedding of CD16 from individual NK cells with the functional properties (cytolysis and secretion of cytokines) of each cell.
3.3 Materials & Methods

3.3.1 Cells and stimulations

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare) from the whole blood of healthy donors (Research Blood Components, Boston, MA; or the Brigham & Women’s Hospital (BWH) PhenoGenetic Project, Boston, MA) and were cryopreserved in 90% heat inactivated fetal bovine serum (FBS; PAA Laboratories) with 10% dimethyl sulfoxide (DMSO; Electron Microscopy Science). Before use, cryopreserved PBMCs were thawed, washed with complete media (RPMI-1640 (Mediatech) supplemented with 2 mM l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM HEPES buffer (all from Mediatech), and 10% FBS), and then incubated (37°C, 5% CO₂) in complete media. Cells were either rested for 1 h in media only or were activated for 20 h with 100 U/mL of recombinant human IL-2 (PeproTech).

P815 and K562 cells were used as target cells. P815 cells are a murine mastocytoma cell line, and K562 cells are a major histocompatibility complex (MHC) class I-deficient chronic myelogenous leukemia cell line. Both cell lines were purchased from ATCC and maintained in complete media without antibiotics. Target cells were split the day before the assay. Immediately before preparing the target cells for the single-cell assays, dead target cells and cellular debris were removed from the cultures by density centrifugation using Ficoll-Paque PLUS. To stimulate NK cells through the CD16 receptor and induce ADCC, P815 target cells were coated with rabbit anti-mouse lymphocyte antibody (1 µL per 100,000 cells; Accurate Chemical & Scientific Corporation) for 30 min and then washed with complete media.
3.3.2 Measurement of surface-expressed CD16 and NKG2D by flow cytometry after bulk stimulation with target cells

PBMCs (10^6/well) and target cells (10^5/well) were added in a total of 200 uL to a U-bottom 96-well plate. The plate was centrifuged for 1 min at 10 g to bring the cells to the bottom. Cells were incubated for the indicated periods of time (37°C, 5% CO₂) and then stained with antibodies against surface markers or isotype controls (anti-CD3-FITC, anti-CD14-FITC, anti-CD19-FITC, anti-CD56-PerCPCy5.5, anti-CD16-BV421, anti-NKG2D-PE, BV421 IgG1 κ isotype control, PE IgG1 κ isotype control). Antibodies are described in further detail in the Supplementary Methods (Appendix B.1.1). Staining was performed in media at 4°C for 30 min. Viability was assessed using the LIVE/DEAD Fixable Aqua Stain (Life Technologies). Cells were then washed in media, suspended in fixation buffer (1% paraformaldehyde (Electron Microscopy Sciences) in phosphate-buffered saline (PBS)), and acquired on a BD LSR Fortessa (BD Biosciences) flow cytometer. Compensation was performed using single-stained beads (Quantum Simply Cellular anti-mouse IgG beads; Bangs Laboratories). CD56dim NK cells were gated as dead−CD3−CD14−CD19−CD56dim lymphocytes.

3.3.3 Nanowell-based assays to measure the shedding of CD16 and functional activity of individual NK cells

Preparation of cells

CD56dim NK cells were isolated from PBMCs by flow sorting. PBMCs were stained with anti-CD3-FITC, anti-CD14-FITC, anti-CD19-FITC, and anti-CD56-APC in media at 4°C for 30 min. Antibodies are described in further detail in the Supplementary Methods (Appendix B.1.1). After washing, cells were resuspended in PBS with 5% FBS, filtered, and sorted by gating on CD3−CD14−CD19−CD56dim lymphocytes (BD FACS Aria III, BD Biosciences). Compensation was performed using single-stained beads. Sorted CD56dim NK cells were rested in media for 1 h at 37°C and then stained
with the proliferation dye eFluor 670 (2 μM in PBS for 5 min at 37°C, eBioscience).

Target cells were labeled with either CellTrace Violet (CTV; 2 μM in PBS for 20 min at 37°C) or CellTracker Red (CTR; 2.5 μM in media for 20 min at 37°C) (both from Life Technologies) as indicated in the text. For experiments in which both antibody-coated P815 and K562 cells were used on the same array, the P815 cells were labeled with CTV and the K562 cells were labeled with CTR. For experiments with a single type of target on the array, both targets were labeled with CTV. Labeling was performed prior to density centrifugation using Ficoll-Paque PLUS and (in the case of the P815 cells) coating with antibody.

**Video microscopy and microengraving**

Arrays of 50 μm cubic nanowells were prepared as described in Appendix D.1.1, with the following modifications: First, arrays were designed to include 121 wells per field of view (as opposed to 49); the resulting arrays contained 83,490 wells. Second, the poly(dimethylsiloxane) (PDMS) was cured overnight. Third, the arrays were collected into media immediately after plasma treatment.

Labeled NK cells and target cells were adjusted to a concentration of 10⁶/mL, combined and mixed, and then deposited onto the array (~300 μL). After letting the cells settle by gravity for 5 min, arrays were gently washed with media containing SYTOX green (nucleic acid stain to identify dead cells, 1 μM; Life Technologies) and 0.02% human serum (containing IgG; to facilitate registration of the microarray of secreted proteins). The array was then sealed with a glass slide coated with capture antibodies against IgG (Life Technologies / ZyMAX), IFN-γ (Mabtech), and CCL4 (R&D Systems) (prepared as described in Appendix D.1.3) and clamped in a microarray hybridization chamber (Agilent).

The sealed array was removed from the chamber after 20 min at room temperature and transferred to a stage-top incubator (TOKAI HIT) mounted on an automated inverted epifluorescence microscope (Axio Observer; Carl Zeiss, 10×/0.3 objective with a 0.63× demagnifying lens) fitted with an EM-CCD camera (ImagEM, Hamamatsu). A subsection of the array (>35,000 wells) was imaged at 8 min intervals for 6 h. The
array remained sealed with the glass slide during the imaging, allowing the collection of secreted proteins (microengraving) and also ensuring that the contents of each well (both cells and secreted molecules) were physically isolated from neighboring wells.

Immediately after the last image was collected, the sealed array was removed from the stage-top incubator and placed in a 4-well dish on ice for 1 h. This step was necessary to reduce the number of NK cells that were lost when the array was subsequently unsealed. The 4-well dish was then centrifuged (340 g, 1 min),¹ and the glass slide (containing the microengraved proteins) was carefully removed from the array of nanowells under ice-cold media. The microengraved slide was then processed with detection antibodies (anti-IgG-AF700, Jackson Immunoresearch; anti-IFN-γ-AF594, Mabtech; anti-CCL4-AF647, R&D Systems) as described in Appendix D.1.3 and imaged on a commercial microarray scanner (GenePix 4400A, Molecular Devices).

**On-chip staining for CD16 and imaging cytometry**

On-chip staining for CD16 was performed by gently aspirating excess media from the array of nanowells, applying staining solution (1 test volume of anti-CD16-PerCP (Biolegend, clone 3G8) in 200 µL media), covering the array with a lifter slip (Electron Microscopy Sciences), and then incubating for 1–2 h at 4°C. The lifter slip was then removed under ice-cold PBS; the array was left in the PBS for 15–20 min to allow unbound antibody to diffuse away. Excess PBS was aspirated and the array was covered with a lifter slip. The array was then imaged on the microscope. Compensation was performed by imaging single-stained cells and beads (in a 96-well plate) in each of the relevant fluorescent channels.

**3.3.4 Data analysis**

Flow cytometry data were analyzed with FlowJo (Tree Star). Statistical analyses were performed with Prism 5 (GraphPad Software) or R (version 3.0.2). The protein

¹NK cells sometimes crawl onto the surface of the glass slide during the course of the incubation. This step was performed in an attempt to bring these cells down back down into the wells.
arrays produced by microengraving were analyzed using commercial image processing software (GenePix Pro 7, Molecular Devices). The microscopy images were processed with a custom-written script based on ImageJ to segment the cells and extract the fluorescence intensities. After this initial step of data extraction, the data from the video microscopy portion of the assay were analyzed using custom-written MATLAB (R2013b; MathWorks) scripts. The surface marker data (from the imaging cytometry performed at the conclusion of the nanowell-based assay) were analyzed using a custom-written script based on MATLAB and R.

3.4 Results

3.4.1 CD16 is shed from the surface of NK cells after bulk stimulation with target cells

To assess the magnitude and kinetics of the shedding of CD16 from the surface of NK cells, we incubated IL-2-activated (100 U/mL, 20 h) PBMCs with target cells for 0.5–4 h and then quantified the amount of CD16 remaining on the surface of CD56\textsuperscript{dim} NK cells by flow cytometry. We used antibody-coated P815 target cells to stimulate NK cells through CD16, and K562 target cells, which express NKG2D ligands, to stimulate NK cells through NKG2D. Stimulation of NK cells with antibody-coated P815 target cells resulted in a significant and rapid decrease of surface-expressed CD16 (Figure 3-1A). In the absence of stimulation, 80% of NK cells were CD16\textsuperscript{hi}. After 30 min of stimulation with antibody-coated P815 target cells, only 9% of the NK cells remained CD16\textsuperscript{hi}, and by 4 h, <1% remained CD16\textsuperscript{hi}. Stimulation with K562 target cells also resulted in a rapid decrease in surface-expressed CD16, but

\footnote{2The automated image processing software (Crossword) that was recently developed by our lab\textsuperscript{159} for the analysis of protein microarrays could not be used here. Crossword relies heavily on the presence of a clearly defined signal in the background channel. Microengraving without constant clamping produces a clearly defined signal in the background channel, but this signal is often slightly shifted (appearing as a “double-print”), making it difficult for Crossword to function properly. The shift does not occur in the cytokine channels, which makes it straightforward to semi-automatically align the analysis grid in GenePix Pro 7.}

\footnote{3Including CD16\textsuperscript{med} cells, 89% were CD16\textsuperscript{+}.}
to a much smaller extent than that observed after stimulation with antibody-coated P815 target cells: 57% remained CD16\textsuperscript{hi} after 4 h (Figure 3-1B).

We also assessed changes in the surface expression of NKG2D following stimulation with target cells. In the absence of stimulation, 99% of CD56\textsuperscript{dim} NK cells were NKG2D\textsuperscript{+}. The expression of NKG2D remained unchanged following stimulation with antibody-coated P815 cells (Figure 3-1C) but decreased gradually upon stimulation with K562 cells (Figure 3-1D). After 30 min of stimulation with K562 target cells, 96% of the NK cells remained NKG2D\textsuperscript{+}, and by 4 h, 84% still remained NKG2D\textsuperscript{+}. These results suggest that “cis” downregulation was greater than “trans” downregulation: CD16 was downregulated the most by stimulation through CD16 (antibody-coated P815 target cells) and NKG2D was downregulated the most by stimulation through NKG2D (K562 target cells).

It has previously been shown that IL-2 induces the shedding of CD16 from NK cells when high doses (400–500 U/mL) and/or long activation times (up to 4 d) are used.\textsuperscript{153,154} At the dose and activation time used in our experiments (100 U/mL, 20 h), however, we did not observe a significant decrease in the levels of CD16 expressed on the surface of NK cells in the absence of stimulation with target cells. Of the CD56\textsuperscript{dim} NK cells, 81% were CD16\textsuperscript{hi} in the absence of activation with IL-2 and 80% were CD16\textsuperscript{hi} after activation with IL-2. After stimulation with antibody-coated P815 target cells, IL-2-activated CD56\textsuperscript{dim} NK cells downmodulated CD16 more rapidly than resting CD56\textsuperscript{dim} NK cells (Figure B-1). After stimulation of resting NK cells with K562 target cells, we observed a decrease in surface-expressed CD16 that was similar to that observed in Ref. 153 using similar conditions.

Together, these results demonstrated that a large fraction of both resting and IL-2-activated NK cells rapidly and completely shed CD16 from their surface in response to bulk stimulation with antibody-coated P815 target cells, and that a smaller fraction shed CD16 from their surface in response to bulk stimulation with K562 target cells.
Figure 3-1: Expression of CD16 and NKG2D on CD56\textsuperscript{dim} NK cells after stimulation with target cells in bulk. IL-2-activated PBMCs were incubated with (A, C) antibody-coated P815 target cells or (B, D) K562 target cells for 0, 0.5, 1, 2, or 4 h and then stained, fixed, and acquired by flow cytometry. CD56\textsuperscript{dim} NK cells were gated as CD3\textsuperscript{−}CD14\textsuperscript{−}CD19\textsuperscript{−}CD56\textsuperscript{dim} lymphocytes.
3.4.2 CD16 is shed from the surface of NK cells after stimulation with single target cells in nanowells

To precisely quantify and control the number of target cells that each NK cell was exposed to, we used the platform that was introduced in Chapter 2 to isolate individual NK cells and target cells in nanowells. We flow sorted IL-2-activated CD56\textsuperscript{dim} NK cells, labeled them with the proliferation dye eFluor 670 to allow unambiguous identification, and then co-loaded NK cells and target cells (CTV-labeled antibody-coated P815 cells and CTR-labeled K562 cells) onto the array of nanowells. The array was sealed with a glass slide and incubated for 6 h, during which time the cellular occupancy of each well was measured by imaging cytometry. To quantify the amount of CD16 remaining on the surface of each NK cell at the conclusion of the 6 h incubation, we removed the glass slide, stained the array with CD16-PerCP, and performed imaging cytometry.

Single NK cells residing in wells without any target cells maintained high levels of CD16, but the expression of CD16 was drastically reduced on NK cells in wells containing antibody-coated P815 target cells or both types of target cells (Figure 3-2A). NK cells in wells with K562 cells also exhibited a slight but significant decrease in the expression of CD16. These results were consistent when the analysis was further restricted to only include wells with exactly one target cell (Figure 3-2B). Across three independent donors, 89% ± 3% of NK cells in wells with no target cells were CD16\textsuperscript{+} (consistent with the baseline frequency of CD16\textsuperscript{+} NK cells in the CD56\textsuperscript{dim} subset), whereas only 22% ± 4% of NK cells in wells with 1–3 antibody-coated P815 cells were CD16\textsuperscript{+} at the end of the assay (Figure 3-3). Together, these results demonstrated that engagement of a single antibody-coated target cell is sufficient to induce the shedding of CD16 from the majority of CD56\textsuperscript{dim} NK cells.
Figure 3-2: Expression of CD16 on IL-2-activated CD56^{dim} NK cells after stimulation with target cells in nanowells. NK cells were flow sorted from IL-2-activated PBMCs (gating on CD3^-CD14^-CD19^-CD56^{dim} lymphocytes), labeled with eFluor 670, and co-loaded onto an array of nanowells with a mixture of K562 and antibody-coated P815 target cells. After 6 h, cells were stained on the array for CD16-PerCP and imaged. (A) The expression of CD16 was analyzed on NK cells in wells that contained one NK cell and at least one target cell of the indicated type. From left to right, the number of single NK cells analyzed was: 2064 (no target), 958 (K562 only), 601 (P815 only), 550 (both targets). With the exception of the P815 v. both targets comparison (no significant difference), the levels of CD16 were significantly different in all comparisons (**P < 0.01, Kruskal-Wallis test followed by Dunn’s post-test). (B) Events were further gated to include only single NK cells in wells with exactly one target cell of the indicated type. From left to right, the number of single NK cells analyzed was: 2064 (no target), 714 (one K562), 477 (one P815), 198 (one of each target). With the exception of the empty v. K562 comparison (**P < 0.01), significance levels were the same as in (A). Results are representative of 2–3 donors and 2–5 experiments.
Figure 3-3: The majority of NK cells shed CD16 when co-incubated with target cells in nanowells. IL-2-activated NK cells were co-incubated on arrays of nanowells with either K562 cells or antibody-coated P815 cells for 6 h. The frequency of CD16+ NK cells was analyzed by staining for CD16 and imaging the cells on the array at the conclusion of the incubation. Only wells containing a single NK cell were included in the analysis. Bars indicate the median of three donors. A single type of target cell was used per array, resulting in six measurements of empty wells across the three donors. ***$P < .001$, one-way analysis of variance (ANOVA) followed by Tukey’s post-test.
Figure 3-4: Distribution of contact durations between NK cells and target cells. Interactions between IL-2-activated NK cells and target cells were tracked (8 min/frame) over the course of a 6 h co-incubation in nanowells. The longest continuous period of contact (left) and cumulative time in contact (right) were scored for single NK cells in wells with the indicated target cells. Only NK cells that had at least one period of contact with a target cell were included in the analysis. Results are representative of three donors.

3.4.3 CD16 is shed with a probability that depends on the duration of contact with a target cell and the baseline activation state of the NK cell

Given that a single engagement with a target cell could induce the shedding of CD16, we next sought to understand the kinetics by which this process occurs. We used video microscopy to track the interactions between IL-2-activated NK cells and target cells on the array of nanowells during the 6 h incubation. This approach enabled us to make correlated measurements of dynamic interaction parameters and end-point expression of CD16 for thousands of single NK cells on each array. NK cells exhibited a wide distribution of contact times (Figure 3-4). NK cells spent longer in contact with antibody-coated P815 target cells than with K562 target cells, both in terms of their longest continuous period of contact (Figure 3-4, left) and their cumulative time in contact (Figure 3-4, right).
Figure 3-5: **Amount of CD16 remaining on the surface of NK cells after varying durations of contact with target cells.** Interactions between IL-2-activated NK cells and target cells were tracked by video microscopy (8 min interval, 6 h duration) to measure the longest continuous period that each NK cell spent in contact with either (A) antibody-coated P815 target cells or (B) K562 target cells. Cells on the array were then stained for CD16 and imaged again. Each point represents a single NK cell. Only NK cells that had at least one period of contact with a target cell are shown \((n = 2001\) cells (A), 2596 cells (B)). Results are representative of three donors.

The amount of CD16 remaining on the surface of NK cells after interactions with antibody-coated P815 cells was inversely related to the amount of time that they spent in contact with the target (Figure 3-5A). Cells that formed only short contacts (<30 min) retained a high level of surface-expressed CD16, whereas the vast majority of cells that engaged a target for a prolonged period of time (>120 min) displayed a complete loss of CD16. NK cells interacting with K562 target cells were much less likely to form prolonged contacts or to shed CD16 (Figure 3-5B).

To further investigate the correlation between contact time and the shedding of CD16, we binned the NK cells by their longest continuous period of contact with a target cell and then calculated the fraction of NK cells that retained expression of CD16 (Figure 3-6). This analysis was performed for three donors using IL-2-activated NK cells; for one of these donors, we also collected a matched dataset using resting NK cells. When antibody-coated P815 cells were used as the targets, the fraction of
NK cells retaining expression of CD16 declined sharply as a function of the longest duration of contact (Figure 3-6A). This decline was more rapid for IL-2-activated NK cells than for resting NK cells. For example, of the NK cells with a longest period of contact of 100 min, ~50% of the resting NK cells expressed CD16 at the end-point of the assay, whereas only ~20% of the IL-2-activated NK cells did. When K562 cells were used as the targets, the fraction of NK cells retaining expression of CD16 declined gradually as a function of the longest duration of contact; again, the decline was more rapid in IL-2-activated NK cells than in resting NK cells (Figure 3-6B). When antibody-coated P815 cells were used as the targets, almost every NK cell (both IL-2-activated and resting) that was in continuous contact with a target cell for >300 min shed CD16. When K562 cells were used as the targets, however, the fraction of NK cells that had shed CD16 did not reach a plateau within the duration of the 6 h assay, suggesting that the likelihood of shedding CD16 would continue to increase in NK cells that remained in contact with a K562 target for longer than 6 h. Similar trends were observed when cumulative contact time (rather than longest continuous period of contact) was considered (Figure B-2). Together, these results demonstrated that NK cells that spend a longer period of time in contact with a target cell are more likely to shed CD16, and that for a given duration of contact, shedding is more likely in NK cells that are IL-2-activated than in those that are resting.

3.4.4 Bystander NK cells lose CD16 more frequently than expected by chance

Monitoring single IL-2-activated NK cells in wells with antibody-coated P815 cells demonstrated that NK cells shed CD16 with a probability that is well predicted by their duration of contact with a target. We next sought to address whether CD16 is also shed from “bystander” NK cells in the local vicinity. This task is experimentally challenging, because in wells with multiple NK cells, the measurements of contact (by video microscopy) and the measurements of CD16 can be correlated at the level of
Figure 3-6: NK cells shed CD16 with a probability that depends on their longest duration of contact with a target cell and their activation state. The fraction of NK cells that retained expression of CD16 is plotted as a function of the duration of their longest continuous period of contact with (A) antibody-coated P815 target cells or (B) K562 target cells. NK cells were either IL-2-activated (orange, combined data from three donors) or resting (green, data from one donor, who was also included in the IL-2-activated data set). Trend lines were estimated by LOESS regression; shading represents the 95% confidence interval.

Each individual well but not at the level of each cell in the well. Furthermore, the time-interval used in these experiments (8 min/frame) was not short enough to enable the accurate tracking of multiple highly motile NK cells when their paths intersected.

We instead approached the question of CD16 shedding from bystander NK cells by using a simple application of the binomial distribution. The binomial distribution states that the probability of observing \( k \) successes in \( N \) independent trials, each of which succeed with a probability of \( p \), is:

\[
P(k) = \binom{N}{k} p^k (1-p)^{N-k} \tag{3.1}
\]

Adapted to the question of CD16 shedding, each NK cell in a well can be treated as a trial that succeeds if the NK cell is CD16\(^+\) at the end of the assay and fails if the

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\( ^4 \)For example, consider a well with two NK cells, Cell A and Cell B. In the video microscopy portion of the assay, we track the dynamic properties and Cell A and Cell B. We then stain for CD16 and image the well again, but because the cells may have shifted between the last image of the time-lapse sequence and this image, it is not possible to unambiguously tell which surface marker phenotype corresponds to Cell A and which corresponds to Cell B.
NK cell is CD16+. We used the data from wells containing a single IL-2-activated NK cell and 1–3 antibody-coated P815 target cells to calculate \( p_{\text{CD16}^+} \), the probability of success for any individual NK cell. We then applied the binomial distribution to calculate the predicted number of CD16\(^+\) NK cells (i.e., \( k \) successes) in wells containing two or three NK cells (i.e., \( N = 2 \) or 3 trials) and 1–3 target cells.

The predicted frequencies of wells in which all NK cells remained CD16\(^+\) matched the experimentally observed frequencies quite closely (Figure 3-7, gray dots). In these wells, presumably none of the NK cells engaged a target long enough to induce the shedding of CD16. In contrast, the observed frequencies of wells in which some or all of the NK cells were CD16\(^-\) deviated from the predictions. Wells in which all NK cells were CD16\(^-\) (Figure 3-7, red dots) were observed more frequently than expected, whereas “mixed” wells containing some CD16\(^+\) NK cells and some CD16\(^-\) NK cells (Figure 3-7A, blue dots; Figure 3-7B, blue and green dots) were observed less frequently than expected. These deviations suggest that when one NK cell sheds CD16, other nearby NK cells have an increased likelihood of shedding CD16 compared with when they are in isolation. In contrast, killing did not occur in a cooperative manner (Figure B-3), similar to our previous findings using K562 target cells.\(^99\) Although the frequencies of wells with mixed states of occupancy were lower than predicted, there were still many wells that exhibited these mixed states (for example, 22\% ± 6\% of the wells containing two NK cells had one CD16\(^+\) NK cell and one CD16\(^-\) NK cell). Together, these findings suggest that there is a mechanism by which nearby NK cells shed CD16 in a coordinated manner, but that shedding of CD16 from neighboring cells is not an all-or-none event, even when NK cells are confined in close proximity.

3.4.5 NK cells that perform effector functions shed CD16, but many cells that shed CD16 do not perform common effector functions

In addition to inducing the shedding of CD16, engagement with target cells can stimulate NK cells to perform effector functions such as cytolysis and the secretion of
Figure 3-7: **Bystander NK cells shed CD16 more often than expected by chance.** Wells containing (A) two or (B) three IL-2-activated NK cells and 1–3 antibody-coated P815 target cells were included in the analysis. The binomial distribution was used to predict the fractions of wells containing the indicated numbers of CD16⁺ NK cells, as described in the text. Predicted fractions were then compared with the experimentally observed fractions. Data from three donors are shown. In (A), all three of the actual (observed) fractions differed significantly (at the $\alpha = 0.05$ level) from the predicted fractions according to Pearson’s chi-squared test, corrected for multiple comparisons with the Benjamini-Hochberg method of controlling the false discovery rate (FDR). In (B), none of the comparisons met the significance threshold after correcting for multiple comparisons. The total number of wells analyzed from each donor/experiment was (A) 216, 401, and 403; (B) 19, 63, and 33.
cytokines. To characterize the relationship between target-induced shedding of CD16 and the functional activity of each cell, we made correlated measurements of cytolytic activity (assessed during the video microscopy portion of the assay), the secretion of IFN-γ and CCL4 (assessed by microengraving at the same time as performing video microscopy), and the end-point expression of CD16. We found that the NK cells that were CD16− at the conclusion of the co-incubation with target cells were almost exclusively responsible for the performance of effector functions when either antibody-coated P815 (Figure 3-8A) or K562 (Figure 3-8B) cells were used as the targets. NK cells that remained CD16+ were almost entirely devoid of functional activity when antibody-coated P815 cells were used as the targets, suggesting that only NK cells that shed5 CD16 were functionally active in this condition. In contrast, a small but reproducible fraction of NK cells that remained CD16+ exhibited effector functions when K562 cells were used as the targets, suggesting that for this stimulation, a minority of NK cells could perform effector functions without the concurrent loss of CD16.

Finally, we asked whether all NK cells that actively shed CD16 from their surface in response to stimulation with a target cell also killed the target or secreted IFN-γ or CCL4. This question cannot be answered directly from the observed data due to the fact that ∼10% of the NK cells in wells with target cells will be CD16− at baseline. Thus, from the end-point measurement of the expression of CD16, we cannot definitively say whether any given CD16− cell in a well with target cells began the assay CD16+ and then actively shed CD16, or whether it simply was CD16− to begin with. However, for these wells, we can estimate the fraction of CD16− NK cells that actively shed CD16 (call this fraction A) by using the observed baseline fraction of CD16− NK cells on each array (B, from the wells containing an NK cell but no target) and the observed fraction of single NK cells (in wells with targets) that were

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5Because CD16 mediates the recognition of antibody-coated P815 cells, we can infer in this case that NK cells that were CD16− at the conclusion of the co-incubation, but that exhibited effector functions, actively shed CD16 (as opposed to being from the ∼10% population of NK cells that were CD16− at baseline).
Figure 3-8: **Functionally active NK cells shed CD16.** The cytolytic and secretory activity of single IL-2-activated NK cells in wells with 1–3 (A) antibody-coated P815 or (B) K562 target cells were measured and segregated based on whether the NK cell expressed CD16 at the conclusion of the 6 h co-incubation (mean percentages for each group are indicated). Paired data from three donors are shown. The percentage of cells that were positive for each function were corrected for the background frequencies, which were calculated from wells with 0 NK cells and 1–3 target cells (for background lysis) or wells with an NK cell but no targets (for background secretion). Note difference in scale between graphs. *$P < .05$, paired t-test.
CD16− at the conclusion of the assay (call this fraction $C$):

$$A = 1 - \frac{B}{C}$$ (3.2)

When we compared this estimated fraction of actively shedding CD16− NK cells to the fraction of CD16− NK cells that exhibited any of the three effector functions measured (cytolysis, secretion of IFN-γ or CCL4), we found that shedding occurred more frequently than the performance of effector functions when antibody-coated P815 cells were used as targets (Figure 3-9A), but that shedding and effector function occurred at similar frequencies when K562 cells were used (Figure 3-9B). A significant portion (28% ± 7%) of NK cells that shed CD16 in response to antibody-coated target cells failed to demonstrate an effector function, whereas this portion was only 7% ± 4% when K562 cells were used as targets. Both types of target cell induced significantly more NK cells to shed CD16 than to mediate cytolysis (Figure B-4). Overall, these results demonstrated that the majority of IL-2-activated NK cells that perform common effector functions upon encounter with a target cell also shed CD16, but that cells that shed CD16 do not necessarily lyse the target or secrete IFN-γ or CCL4 during the course of the 6 h assay.

### 3.5 Discussion

Dynamic changes in the expression of cellular surface receptors—and the functional correlates and consequences of these changes—shape many immunological processes. Receptor modulation is often induced by stimulatory signals that are received from direct cell-cell interactions, but it is experimentally challenging to correlate the stimuli that individual cells receive from discrete periods of contact with the cells’ resulting changes in receptor expression and functional outcomes. The nanowell-based platform presented here offers one approach to making these types of correlated measurements on single cells. In this approach, large numbers (thousands) of individual cells are dynamically tracked as they participate in cell-cell interactions, and the properties...
Figure 3-9: Not all NK cells that shed CD16 perform common effector functions. The frequency of IL-2-activated NK cells that actively shed CD16 (calculated as described in the text) in response to engaging an (A) antibody-coated P815 or (B) K562 target cell was compared with the frequency with which these cells mediated any of the effector functions measured (cytolysis or the secretion of IFN-γ or CCL4). Paired data from three donors are shown. *P < .05, paired t-test.

of these interactions—as well as their functional outcomes—are directly linked to the surface marker phenotype of the cell. Furthermore, the receptor of interest (e.g., CD16) is not perturbed by antibody staining until after the functional analysis.

We investigated the downmodulation of CD16 from the surface of NK cells in response to stimulation from contact with target cells. The patterns of downmodulation that we observed in the nanowell assays were consistent with those observed after bulk incubation with target cells and analysis by flow cytometry. In both cases, stimulation with antibody-coated P815 target cells resulted in a more complete loss of CD16 than did stimulation with K562 target cells, which is in agreement with previous reports\textsuperscript{156} and with the observation that signaling through CD16 (triggered here by antibody-coated P815 cells) provides stronger activation than signaling through other receptors such as NKG2D\textsuperscript{103,104} (triggered here by K562 cells).

After bulk incubation with K562 target cells, CD16 was downregulated in a bimodal fashion (the majority of NK cells either retained high levels of CD16 or completely shed CD16), whereas NKG2D was downregulated in a unimodal fashion (the
entire distribution shifted to the left). Despite providing strong activating signals, stimulation with antibody-coated P815 target cells did not alter the expression of NKG2D on the surface of NK cells. Differences in the target-induced downregulation of CD16 and NKG2D may reflect the different mechanisms by which each receptor is removed from the cell surface. Although both signaling from and downregulation of CD16 and NKG2D share nodes such as phosphatidylinositol 3-kinase (PI3K), the loss of CD16 occurs mainly by proteolytic shedding and can be induced by a wide range of stimuli, whereas the loss of NKG2D is thought to involve clathrin-dependent endocytosis and is induced by ligand binding to NKG2D.

Both the bulk and single-cell assays demonstrated that the speed and magnitude of CD16 shedding from the surface of NK cells is affected by the activation state of the NK cell. In the bulk assays, CD16 shedding was more rapid from IL-2-activated NK cells than from resting NK cells. Similarly, shorter periods of contact with target cells were required to induce CD16 shedding in IL-2-activated NK cells than in resting NK cells. The observation that activation with IL-2 increases the speed (or lowers the threshold) of CD16 shedding in response to contact with target cells is consistent with previous reports that IL-2 alone (if used at higher doses and/or longer treatment periods than were used here) can induce CD16 shedding. The influence of IL-2-activation on CD16 shedding and the performance of effector functions is important to consider in the context of cancer therapies that combine rituximab (a monoclonal antibody that induces ADCC) with IL-2.

By monitoring interactions between individual NK cells and target cells, we demonstrated that prolonged contact with a single antibody-coated P815 target cell induced the shedding of CD16 in the majority of NK cells. Many of these cells also performed effector functions such as cytolysis of the target or secretion of IFN-γ or CCL4. These results are consistent with previous reports showing that target-induced downregulation of CD16 is associated with the production of IFN-γ and degranulation. Many other actively shedding NK cells, however, did not perform any of the three effector functions that we measured (at least during the 6 h assay). Previous studies have also shown that the fraction of NK cells that shed CD16 is higher than
the fraction that degranulate or produce IFN-γ. The single-cell measurements presented here extend these previous findings (which used bulk incubations of NK cells and targets) to demonstrate that while common effector functions arising from discrete encounters with individual target cells are concentrated within the subset of NK cells that actively shed CD16, not all actively shedding NK cells perform common effector functions.

Single-cell measurements (e.g., by flow cytometry) that are performed after bulk co-incubation of NK cells and target cells enable the phenotype and function of each individual cell to be resolved, but these types of measurement do not enable the properties of neighboring cells to be linked in a way that reflects potential intercellular interactions. In contrast, the nanowell-based platform presented here enables measurements that are made on both a “per well” and a “per cell” basis. Thus, the expression of CD16 on each NK cell can be compared with that of neighboring NK cells residing within the same nanowell. By measuring the distribution of CD16 states in wells containing multiple NK cells (and at least one antibody-coated P815 target cell), we found that the shedding of CD16 occurred in a more coordinated fashion than would be expected by chance. In other words, wells containing mixed populations of CD16⁺ and CD16⁻ NK cells were observed less frequently than expected, while wells containing all CD16⁻ NK cells were observed more frequently than expected. We did not observe cooperative killing behavior in these cases (similar to our previous findings), which suggests that although “bystander” NK cells have an increased likelihood of shedding their CD16, they do not have significantly increased cytolytic activity.

Several possible mechanisms could contribute to the coordinated loss of CD16 from the surface of neighboring NK cells. Activated NK cells might secrete proteases that act not only on the activated cell, but also on other cells in the local vicinity. The two proteases that have so far been associated with the cleavage of CD16 (ADAM17 and MMP25) are membrane proteins and thus should not operate by this mechanism, but it is possible that secreted proteases also contribute to the shedding of CD16. Alternatively, proteases on the surface of an activated NK cell might
cleave CD16 from the surface of another cell in *trans* if the two cells engage in close physical contact. Shedding in *trans* has been investigated by mixing NK cells with T cells that were transduced with CD16 and FcεRIγ (CD16/γ).\textsuperscript{157} In this setting, shedding occurred predominately from NK cells when the cell mixture was stimulated with plate-bound antibodies against NK cell activating receptors but from CD16/γ-transduced T cells when the cell mixture was stimulated with anti-CD3. Therefore, in this case, shedding was confined to the activated cell type (i.e., shedding in *cis* but not *trans*). However, there are two important differences between this experiment and the ones presented here: first, the stimulations only induced mild downregulation of CD16 (as opposed to the nearly complete downregulation that we observed when using antibody-coated P815 target cells as the stimuli); second, shedding in *trans* mediated by homotypic interactions\textsuperscript{6} between NK cells (as opposed to NK-T cell interactions) would not have been detected by the assay. Therefore, it is possible that shedding in *trans* could occur in other settings. We frequently observed close homotypic interactions between NK cells in the same nanowell, suggesting that activated surface proteases on one cell might be able to cleave CD16 from the surface of another cell.

Coordinated loss of CD16 from the surface of neighboring NK cells could also occur by a variety of indirect mechanisms. It is possible that activated (shedding) NK cells secrete cytokines or participate in contact-mediated interactions with other NK cells that increase the likelihood that these other cells will also shed (e.g., by lowering the threshold of additional activation that is required for shedding or by bringing other NK cells near the target(s)\textsuperscript{98}). We did not observe cooperative cytolytic behavior in wells with multiple NK cells, however, which suggests that NK-to-NK influences on shedding and on effector functions are at least somewhat disconnected. From the current set of data we cannot definitively say which—if any—of the possible mechanisms discussed above account for the coordinated behavior of CD16 shedding, but future experiments could be performed to address each of these possibilities.

\textsuperscript{6}Homotypic interactions between NK cells can be mediated by a variety of surface molecules and are thought to play a role in proliferation and the acquisition of effector functions.\textsuperscript{165,166}
Although we focused here on the shedding of CD16, other surface receptors that play important roles in the functional properties of NK cells are known to be modulated by proteolytic shedding of their ectodomain. One such receptor is CD62L (L-selectin), which, like CD16, is cleaved and shed by ADAM17. Given its function as a homing receptor targeting secondary lymphoid tissues, it is interesting to speculate how activation-induced downregulation of CD62L might alter the tissue distribution of activated NK cells \textit{in vivo}. CD62L has been identified as a marker of polyfunctional NK cells that respond strongly to stimulation with both cytokines and target cells, and it has been suggested that CD56\textsuperscript{dim}CD62L\textsuperscript{+} NK cells are at an intermediate state of maturation between the CD56\textsuperscript{bright} and the CD56\textsuperscript{dim}CD62L\textsuperscript{−} subsets. By enabling the correlation of functional activity, interaction dynamics, and ectodomain shedding, the platform presented here offers a new approach to investigating the unique functional properties of the CD56\textsuperscript{dim}CD62L\textsuperscript{+} subset.

The shedding of CD62L is also of interest as a marker of antigen-specific T cell activation through the T cell receptor (TCR). CD62L is highly expressed on naïve T cells but is shed rapidly upon stimulation through the TCR. In this context, the speed and extent of CD62L shedding has been shown to reflect the strength and duration of TCR signaling both \textit{in vitro} and \textit{in vivo}. These shedding trends are similar to our observation that the speed and extent of CD16 shedding from NK cells is correlated with the strength (i.e., strong stimulation presented by antibody-coated P815 cells or weaker stimulation presented by K562 target cells) and duration of stimulation with target cells, as well as by the activation state (resting or IL-2-activated) of the NK cell. We note that the platform presented here could be extended to monitor CD62L shedding from naïve T cells after encounters with antigen-presenting cells (APCs). Using this approach, antigen-specific activation of T cells could be measured in a way that correlates the rapid shedding of CD62L with subsequent functional responses such as the secretion of cytokines or proliferation.

In summary, we have developed a nanowell-based platform that enables measurement of the kinetics by which and extent to which CD16 is shed from NK cells following discrete encounters with individual target cells. In addition, these measurements
enable correlated analysis of shedding and effector functions and analysis of potential cooperative shedding behaviors amongst small groups of cells. This platform can be extended to investigate the dynamic, functional, and cooperative properties of the shedding (or modulation by other mechanisms) of other surface molecules of interest.
Chapter 4

Single-Cell Analysis of the Dynamic and Functional Properties of Licensed and Unlicensed Natural Killer Cells
4.1 Abstract

It is thought that natural killer (NK) cells are conferred with enhanced functional abilities when they receive signals through inhibitory receptors (IRs) that recognize cognate HLA Class I molecules on host cells. NK cells expressing IRs that recognize host HLA Class I molecules are considered to be functionally “licensed”, whereas NK cells that lack these inhibitory signals are considered to be functionally “unlicensed”. The mechanisms underlying licensing are not well understood, in part because of the large amount of cell-to-cell heterogeneity among NK cells. Furthermore, licensing is thought to affect the early stages of interactions between NK cells and target cells (e.g., conjugation), but it is experimentally challenging to integrate measurements of these early stages of interaction with downstream functional properties and the IR phenotypes of individual NK cells. To address these challenges and characterize the licensing process at the single-cell level, we used nanowell-based assays to measure the dynamic and functional properties of individual IR$^+$ (licensed) and IR$^-$ (unlicensed) NK cells as they interacted with target cells. Both licensed and unlicensed NK cells secreted cytokines with a frequency that increased with the longest duration of contact with a target cell, but unlicensed NK cells were less likely than licensed NK cells to secrete cytokines, even after prolonged contact with a target. These results suggest that there are underlying differences in the characteristics of licensed and unlicensed NK cells that are not completely accounted for by differences in their ability to form an initial engagement with a target cell.

4.2 Introduction

Natural killer (NK) cells express a diverse range of inhibitory receptors (IRs) that recognize human leukocyte antigen (HLA) molecules on healthy cells. These inhibitory signals play a critical functional role by preventing NK cells from attacking healthy cells. Two major HLA-specific IRs on NK cells are NKG2A-CD94 heterodimers (hereafter referred to as NKG2A) and members of the killer immunoglobulin-like receptors
(KIR) class of receptors. NKG2A is expressed on CD56\textsuperscript{bright} NK cells early in development and is gradually lost as the NK cells transition to the CD56\textsuperscript{dim} subset. Conversely, KIRs are not highly expressed on CD56\textsuperscript{bright} NK cells but expression is gradually acquired as the NK cells mature. The complementary patterns of NKG2A and KIR expression ensure that the majority of NK cells express some form of IR at each of their developmental stages.

NKG2A is a C-type lectin receptor that recognizes HLA-E, a non-classical (class Ib) major histocompatibility (MHC) molecule that presents peptides derived from the signal sequences of MHC Class I molecules and delivers inhibitory signals through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on its cytoplasmic domain. The KIR class of receptors contains >12 highly homologous members that are characterized by two or three (2D or 3D, respectively) extracellular Ig-like domains and a long or short (L or S, respectively) cytoplasmic tail. The long cytoplasmic tails contain one or more ITIMs, making them inhibitory receptors (although KIR2DL4 is an exception). The short cytoplasmic tails associate with immunoreceptor tyrosine-based activation motif (ITAM)-containing proteins, making them activating receptors. In general, the inhibitory KIR recognize MHC Class I molecules. Activating KIR bind weakly to the HLA ligand of their inhibitory counterpart, but specific high-affinity ligands of the activating KIRs are for the most part unknown.

In the human population, over 20 KIR haplotypes containing different combinations of the \textit{KIR} genes have been described. KIR2DL4, KIR3DL2, and KIR3DL3 are found in almost every haplotype and are known as “framework” genes, while other \textit{KIR} genes are present in some individuals but absent in others. In addition to variations in haplotype across individuals, there is a significant amount of cell-to-cell variability in the expression of KIR within any one individual. The subset of KIR genes that are expressed in each cell appears to be stochastically controlled by methylation of the KIR promoters. Once the promoter methylation has been established during NK cell development, the pattern of expression remains stable throughout multiple cell divisions. Analysis of 100 NK cell clones derived from two individuals showed that each cell expressed RNA for 2 to 9 KIR and CD94:NKG2 re-
ceptors per cell. This variability means that in a person whose haplotype contains 9 KIR genes, one NK cell might express a subset of 3 KIR genes while another NK cell might express a different subset of 7 KIR genes.

Different inhibitory KIR preferentially bind to different HLA Class I molecules, and each of these binding interactions has a different affinity and associated degree of inhibitory signaling. For example, KIR2DL1 binds group 2 HLA-C molecules (HLA-C2), KIR2DL2 and KIR2DL3 bind group 1 HLA-C molecules (HLA-C1), and KIR3DL1 binds HLA-B molecules of the Bw4 serotype (HLA-Bw4). Because HLA and KIR loci are on separate chromosomes (6 and 19, respectively) and segregate independently, it commonly happens that a subset of NK cells in individuals will express KIRs that do not recognize any of the individual’s HLA molecules. For example, a KIR3DL1+ NK cell in an HLA-Bw4/Bw4 or an HLA-Bw4/Bw6 individual would be able to receive inhibitory signals from the cognate HLA molecules, but the same NK cell in an HLA-Bw6/Bw6 individual would not.

NK cells that do not express any IRs and those that express IRs that do not recognize HLA Class I molecules expressed by the individual are in theory at risk for damaging healthy cells, because these NK cells are unable to receive the inhibitory signals that the healthy self cells present. It is thought, however, that NK cells guard against this undesirable autoreactivity and maintain tolerance in an IR-dependent process known as “licensing”. One model of NK cell licensing posits that during development, NK cells must express an IR that recognizes self-MHC in order to become licensed to kill and secrete cytokines in response to stimulation with target cells. NK cells that do not express such an IR are anergic (unlicensed) unless exposed to an inflammatory environment. The degree of licensing (as measured by functional activity in response to stimulation with HLA-deficient target cells) that each NK cell receives depends on which IRs the NK cell expresses and whether the individual expresses the cognate HLA molecules for those IRs. For example, licensing through NKG2A (binding to HLA-E, which is expressed by all individuals) is weaker than licensing through KIR2DL1, KIR2DL3, or KIR3DL1 when the individual expresses the cognate HLA molecules for these KIR (HLA-C2, HLA-C1, and HLA-Bw4, re-
spectively), and licensing is stronger still in NK cells that express multiple inhibitory KIR (e.g., KIR2DL1\(^+\)KIR3DL1\(^+\) double-positive NK cells in an HLA-C2, HLA-Bw4 individual).\(^{181}\)

The effects of licensing can be observed both by comparing IR\(^+\) and IR\(^-\) NK cells within the same individual, as well as by comparing IR\(^+\) NK cells from individuals with HLA backgrounds that either do or do not contain cognate HLA ligands. Experimental protocols employing pan-IR staining or separation of NK cells (in which NK cells expressing NKG2A, KIR2DL1/2/3, and/or KIR3DL1 are distinguished from those that do not express any of these IRs) have demonstrated that IR\(^+\) NK cells are more functionally active against MHC-deficient target cells (K562 or antibody-coated P815 cells) than IR\(^-\) NK cells are, as assessed by degranulation (CD107a\(^+\)) and the production of interferon-\(\gamma\) (IFN-\(\gamma\)).\(^{182,183}\) Similar functional differences have been demonstrated by comparing NK cells that are IR\(^-\) with NK cells that express a single KIR (with a matched HLA molecule expressed by the individual). For example, stimulation with K562 target cells induces degranulation in a larger fraction of KIR3DL1\(^+\) NK cells than in KIR3DL1\(^-\) NK cells (gated on CD56\(^{dim}\)NKG2A\(^-\)KIR2D\(^-\))\(^1\) from HLA-Bw4 donors.\(^{123}\) Furthermore, KIR\(^+\) NK cells from individuals that express cognate HLA ligands for that KIR are more functionally active against MHC-deficient target cells than the same subset of KIR\(^+\) NK cells from individuals that express HLA ligands that the KIR does not recognize. For example, KIR2DL1\(^+\) NK cells are more responsive when isolated from HLA-C2 individuals than from HLA-C1/C1 individuals,\(^{181,182}\) KIR2DL2\(^+\) and KIR2DL3\(^+\) NK cells are more responsive when isolated from HLA-C1 individuals than from HLA-C2/C2 individuals,\(^{169,181,182}\) and KIR3DL1\(^+\) NK cells are more responsive when isolated from HLA-Bw4 individuals than from HLA-Bw6/Bw6 individuals.\(^{181,184-187}\) At least in some cases, homozygosity for a cognate HLA ligand can increase the licensing effect, as it has been shown that KIR3DL1\(^+\) NK cells from HLA-Bw4/Bw4 individuals are more responsive than those from HLA-Bw4/Bw6 individuals, although both are more responsive than KIR3DL1\(^+\) NK cells from HLA-Bw6/Bw6 individuals.\(^{186}\)

\(^1\)Here, KIR2D encompasses KIR2DL1/2DS1/2DL2/2DS2/2DL3.
The expression of certain KIR-HLA combinations has been shown to influence the outcome of several diseases, including human immunodeficiency virus (HIV).\textsuperscript{188} HIV-infected individuals expressing KIR3DL1 along with HLA-Bw4-80I have a delayed progression to AIDS, and the effect varies with KIR3DL1 allele.\textsuperscript{189} These findings suggest that licensing interactions can contribute to the control of viral infections.

Although the mechanistic basis of licensing is not yet fully characterized, several lines of evidence suggest that functional differences between licensed and unlicensed NK cells arise in part due to differences in their ability to receive activating signals from target cells. A study using a humanized transgenic mouse model in which NK cells are licensed through the expression of KIR2DL3 and its interaction with HLA-C1 (specifically, HLA-Cw3) demonstrated that in licensed NK cells, activating receptors are clustered into nanodomains that are favorable for signaling, whereas in unlicensed NK cells, activating receptors are confined alongside inhibitory receptors in an actin meshwork.\textsuperscript{190} It has also been shown that unlicensed murine NK cells (isolated from \(\beta^{2m-/-}\) mice, which are deficient in MHC Class I and thus do not support licensing interactions) and unlicensed (IR\textsuperscript{−}) human NK cells form fewer stable conjugates with target cells than their licensed counterparts.\textsuperscript{183} This deficiency in forming stable conjugates contributes to the decreased functional activity of unlicensed cells against target cells and arises from impaired “inside-out” signaling from activating receptors to the \(\beta2\) integrin leukocyte function-associated antigen 1 (LFA-1). Inside-out signaling is responsible for switching LFA-1 from a low-affinity state to a high-affinity state, and thus controls the ability of an NK cell to adhere to a target cell via interactions between LFA-1 (on the NK cell) and its ligand intercellular adhesion molecule 1 (ICAM-1; on the target cell). Together, these studies illustrate that NK cells that are able to receive inhibitory signals from cognate HLA molecules are better able to interact with, interrogate, and receive activating signals from target cells.

Here, we adapt and apply the approaches introduced in Chapter 3 to directly monitor how individual licensed (IR\textsuperscript{+}) and unlicensed (IR\textsuperscript{−}) NK cells interact with target cells. We correlate the properties of these interactions with the resulting functional outcomes to demonstrate that licensed and unlicensed NK cells that share certain
dynamic properties of interaction (specifically, duration of contact with a target cell) still differ in their ability to secrete cytokines. We also begin to explore how transcriptional profiling with RNA-Seq can be applied to characterize these differences in more detail.

4.3 Materials & Methods

4.3.1 Nanowell-based assays

Cells and stimulations

Peripheral blood mononuclear cells (PBMCs), P815 target cells, and K562 target cells were prepared as described in Section 3.3.1.

Nanowell-based assays to measure the expression of IRs and functional activity of individual NK cells

Nanowell-based assays were performed as described in Section 3.3.3, but on-chip staining was performed for a panel of IRs instead of (or, in some experiments, in addition to) on-chip staining for CD16. The staining solution was prepared in a total of 200 µL media by adding 1 test volume each of anti-NKG2A-PE, anti-KIR2DL1-PE, anti-KIR2DL2/L3/S2-PE, and anti-KIR3DL1-PE. We did not stain for KIR3DL2, as interactions between this receptor and its cognate HLA ligands (HLA-A3 and HLA-A11) have not been shown to mediate licensing (KIR3DL2 single-positive NK cells are hyporesponsive in HLA-A3 and HLA-A11 individuals). Antibodies are described in further detail in the Supplementary Methods (Appendix C.1.1). Arrays were incubated with the staining solution for 1 h to overnight at 4°C.

For Donor 1 and Donor 2, the complete panel of IRs was used because the KIR and HLA types of these donors were unknown. For Donor 3, anti-KIR2DL2/L3/S2-PE was omitted from the staining panel because this donor was KIR and HLA typed and known to be homozygous for HLA-C2 (therefore KIR2DL2/L3+ NK cells should not receive strong inhibitory/licensing signals due to the absence of HLA-C1).
Flow cytometry to measure the expression of IRs (for comparison with imaging cytometry)

PBMCs ($0.5 \times 10^6$) were stained with both (1) the panel of antibodies that was used to sort the CD56$^{\text{dim}}$ NK cells for use in the nanowell-based assays and (2) the panel of antibodies against IRs that was used in the on-chip imaging cytometry. Antibodies are described in further detail in the Supplementary Methods (Appendix C.1.1). Staining was performed in media at 4°C for 30 min. Cells were then washed in media, resuspended in phosphate-buffered saline (PBS) with 5% fetal bovine serum (FBS), filtered, and acquired by flow cytometry (BD FACSria III, BD Biosciences). CD56$^{\text{dim}}$ NK cells were gated as CD3$^-$$^-$CD14$^-$$^-$CD19$^-$$^-$CD56$^{\text{dim}}$ lymphocytes. Compensation was performed using single-stained beads (Quantum Simply Cellular anti-mouse IgG beads; Bangs Laboratories).

Data analysis

Data were analyzed as described in Section 3.3.4.

4.3.2 Population-level RNA-Seq

Cells

Cryopreserved PBMCs (Ragon Institute HIV-Negative Cohort) were thawed, washed with media, and then incubated (37°C, 5% CO$_2$) for 1.5 h. P815 target cells were split the day before the assay. Immediately before the assay, they were coated with rabbit anti-mouse lymphocyte antibody (1 µL per 100,000 cells; Accurate Chemical & Scientific Corporation) for 30 min and then washed with media.

Prior to labeling and flow sorting, PBMCs ($2 \times 10^6$ cells/mL) were either incubated with antibody-coated P815 target cells ($0.2 \times 10^6$ cells/mL) or with media alone for 4 h.

Surface marker staining and flow sorting

After the 4 h incubation, cells were stained with antibodies against lineage mark-
ers and IRs (anti-CD3-PECy7, anti-CD14-PECy7, anti-CD19-PECy7, anti-CD56-PerCPCy5.5, anti-NKG2A-APC, anti-KIR2DL1-PE, anti-KIR2DL2/L3/S2-PE, and anti-KIR3DL1-BV421). Antibodies are described in further detail in the Supplementary Methods (Appendix C.1.1). Staining was performed in media at 37°C for 15 min. Viability was assessed using the LIVE/DEAD Fixable Aqua Stain (Life Technologies). Cells were washed in media, resuspended in PBS with 5% FBS, and filtered. KIR3DL1+ and KIR3DL1− cells were sorted from the population of dead−CD3−CD14−CD19−CD56dimNKG2A−KIR2DL1−KIR2DL2/L3/S2− lymphocytes (BD FACSAria III, BD Biosciences). Compensation was performed using single-stained beads.

Cells were sorted into 1.5 mL DNA LoBind tubes (Eppendorf) containing 102 µL lysis buffer (100 µL Lysis Buffer RA1, 2 µL tris(carboxyethyl)phosphine (TCEP); Macherey-Nagel NucleoSpin RNA XS RNA isolation kit). After sorting, the tubes were vortexed, centrifuged (300 g, 30 s), and stored at -80°C.

**RNA isolation**

The cell lysates were thawed on ice and RNA was isolated using the NucleoSpin RNA XS RNA isolation kit (Macherey-Nagel) following the manufacturer’s instructions.2 RNA was eluted in 2 × 5 µL RNase-free water and stored at -80°C.

**cDNA synthesis and quantification**

cDNA was synthesized using the SMARTer Ultra Low RNA Kit for Illumina Sequencing (Clontech) following the manufacturer’s instructions3 (1 µL RNA input, 17 cycles long-distance (LD) PCR using the Advantage 2 PCR Kit). cDNA was eluted in 12 µL purification buffer and stored at -20°C. cDNA was quantified using the PicoGreen dsDNA Assay Kit (Life Technologies) and size distributions were verified for a subset of the samples (Fragment Analyzer; Advanced Analytical).

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2Carrier RNA was not used.

3For first-strand cDNA synthesis, 1 µL input RNA was added to a master mix of dilution buffer, RNase inhibitor, and 3’ SMART CDS Primer II A. This protocol differed slightly from the manufacturer’s protocol, which called for first mixing the dilution buffer and RNase inhibitor, then adding the input RNA, and then adding the 3’ SMART CDS Primer II A.
Library preparation and sequencing

Paired-end sequencing libraries were prepared from <1 ng cDNA using the Nextera XT DNA Sample Preparation Kit (Illumina) with the following minor modifications to the manufacturer’s protocol: tagmentation was performed for 10 min at 55°C (instead of 5 min), a 60 s extension was used in the limited-cycle PCR amplification (instead of 30 s), PCR products were cleaned using 0.9× Agencourt AMPure XP beads (Beckman Coulter), and PCR products were eluted in 30 µL of resuspension buffer (instead of 52.5 µL). The bead-based library normalization step was omitted. Libraries were dual indexed using Index 1 (i7) indices N701 – N710 and Index 2 (i5) indices S503 – S506.

Libraries were quantified by qPCR and fragment sizes were verified (Fragment Analyzer; Advanced Analytical) prior to pooling and sequencing (2 × 50 bp paired-end; HiSeq 2000; Illumina). More than 7 × 10^6 reads were obtained for each sample.

Data analysis

Sequencing data were pre-processed using the FASTX Toolkit (Version 0.0.13). Bases with a quality score of less than 28 were trimmed from the end of each sequence (command line usage: fastq_quality_trimmer -t 28 -l 0). Transcript abundances were calculated using RSEM (RNA-Seq by expectation maximization; Version 1.2.6; command line usage: rsem-calculate-expression --paired-end -p 4 --output-genome-bam --calc-ci --phred64-quals) with a reference transcriptome prepared from the UCSC Known Genes database. Differential expression was evaluating using the R/Bioconductor package DESeq.

The GFOLD (generalized fold change) algorithm (Version 1.0.9) was used to rank differentially expressed genes when individual samples were compared. This ranking was used to compare the expression of KIR3DL1 in the cell populations that were sorted as KIR3DL1+ and KIR3DL1− from each donor.
4.4 Results & Discussion

4.4.1 An integrated platform to correlate the expression of inhibitory receptors (IRs) with functional activity and dynamic properties at the single-cell level

We extended the platform introduced in Chapter 2 to enable integrated measurements of functional activity, dynamic properties, and the expression of IRs (NKG2A and KIRs) for individual NK cells. In a typical experiment, NK cells and target cells are co-loaded onto an array of nanowells, and the cytolytic, dynamic, and secretory activity is measured by simultaneously performing time-lapse microscopy and micro-engraving. At the conclusion of the assay, cells are stained on the array for surface markers of interest (in this case, IRs) and acquired by imaging cytometry (Figure 4-1). This order of operations allows the functional properties of cells to be measured prior to perturbing them by staining for the receptor of interest.

Several important improvements and modifications were made to the original platform. First, the number of wells (and hence the upper limit on the number of cellular interaction events of interest) imaged during the time-lapse microscopy portion of the assay was increased from <9,000 to >35,000 while still maintaining an 8 min imaging interval. This increase was enabled by (1) upgrading the microscope with faster acquisition capabilities and (2) altering the design of the array and increasing the field of view of the microscope to enable more wells to be acquired per image (121 compared with 49). Second, an image processing script based on ImageJ was developed to increase the accuracy and speed of segmenting images of cells and extracting their fluorescence intensities in each channel. Third, microengraving was performed at the same time as the time-lapse microscopy (rather than immediately after, as was done previously), enabling direct assessment of the secretion that occurred during

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4 The approaches described in this chapter were also applied to investigate the shedding of CD16 (Chapter 3).

5 The large field of view format was achieved by using a demagnifying lens, which reduced the magnification of each image from $10\times$ to $6.3\times$. NK cells and target cells were still readily identified, even with this decrease in spatial resolution.
Figure 4-1: **Expression of inhibitory receptors on NK cells can be quantified by on-chip staining and imaging cytometry.** The distribution of IR expression on CD56$^{\text{dim}}$ NK cells was measured by flow cytometry (left) and by imaging cytometry (right). Flow measurements were performed by staining an aliquot of PBMCs that came from the same population that was sorted (without labeling IRs) and used in the single-cell functional assays and on-chip imaging cytometry shown on the right. Imaging cytometry was performed at the conclusion of the functional assay by staining on-chip for IRs. Prior to the functional assay, NK cells were stained with eFluor670 to enable unambiguous identification.

the period of observation. Fourth, a protocol was developed (described in Section 3.3.3) to increase the fraction of cells that were retained in the wells between the combined time-lapse imaging/microengraving and the analysis of the expression of IRs by on-chip staining and imaging cytometry. Using this protocol, 79% ± 10% of single NK cells were retained (Figure C-1), thus enabling the assignment of a surface marker phenotype to the majority of cells for which dynamic, cytolytic, and secretory parameters were measured.

Altogether, these improvements made it possible to collect integrated measurements of the dynamic, cytolytic, and secretory properties of thousands of individual NK cells on each array as they interacted with target cells. Previously (Chapter 2), these types of measurements were limited to hundreds of cells per array. This order-of-magnitude increase in the number of cells that could be analyzed, combined with the ability to measure the surface marker phenotypes of cells without perturbing them prior to the functional assays, enabled the investigation of the dynamic and functional properties of IR+ and IR− NK cells. IR− cells comprise a minority of the total NK cell
population (13% ± 6%); thus, the ability to measure thousands—as opposed to hundreds—of cells is crucial. After filtering for quality metrics associated with each measured parameter, on each array we collected complete datasets for an average of 2140 (range 979 to 4815, across 12 experiments) single IR\(^+\) CD56\(^{\text{dim}}\) NK cells interacting with 1–3 target cells, and 225 (range 89 to 583) single IR\(^-\) CD56\(^{\text{dim}}\) NK cells. Thus, the workflow presented here enabled parallel, integrated measurements of large numbers of single IR\(^+\) and IR\(^-\) NK cells.

### 4.4.2 NK cells that express IRs are more functionally active against target cells in nanowells than those that do not

Flow cytometry studies have shown that IR\(^+\) NK cells are more likely than IR\(^-\) NK cells to produce IFN-\(\gamma\) and degranulate when co-incubated in bulk with K562 target cells or antibody-coated P815 target cells.\(^{182,183}\) We therefore investigated whether functional differences between the two subsets of NK cells could be detected using our integrated platform, where individual NK cells are confined in nanowells with a limited number of target cells.

Cytolytic activity was measured by the acquisition of SYTOX (a membrane-impermeable nucleic acid stain) by target cells during the course of the 6 h co-incubation, as originally described in Chapter 2. Concurrent secretory activity was measured by microengraving during the entire period of co-incubation and imaging.\(^6\)

Using this approach, wells that contained NK cells secreting IFN-\(\gamma\) or CCL4 in response to interactions with target cells could be clearly distinguished from empty wells on the same array (Figure 4-2).

When the IR phenotype of each NK cell (measured by imaging cytometry after the functional assays, as in Figure 4-1) was linked to its functional properties, we found that IR\(^+\) NK cells were significantly more likely than IR\(^-\) NK cells to secrete IFN-\(\gamma\) or CCL4 upon contact with antibody-coated P815 target cells in nanowells (Figure 4-6).

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\(^6\)In this method of microengraving, the glass slide coated with capture antibodies is clamped to the array in a metal hybridization chamber, as was done in Chapter 2, but the sealed sandwich is then removed from the chamber and placed on the microscope stage for imaging. Secretion and imaging data can thus be collected concurrently rather than in a staggered manner.
Figure 4-2: Detection of secreted cytokines by microengraving concurrently with time-lapse microscopy. Microengraving was performed for 6 h on a sealed but unclamped array while imaging the array. Histograms of the resulting intensities of IFN-γ (left) and CCL4 (right) are shown for wells that were empty (blue) or that contained a single NK cell and 1–3 antibody-coated P815 target cells. The threshold intensity for positive events (dashed black line) was defined as the median intensity in empty wells plus two standard deviations. Results from Donor 1 are shown and are representative of the other donors. MFI; median fluorescence intensity after correction for local background intensity.
Figure 4-3: **Secretory activity of IR\(^+\) and IR\(^-\) NK cells measured by microengraving.** The secretory activity shown in Figure 4-2 was segregated based on whether the NK cell was IR\(^+\) (green) or IR\(^-\) (red). Results from Donor 1 are shown and are representative of the other donors.

3). These differences in secretory activity were observed in three independent donors (Figure 4-4A) and were maintained when the NK cells were activated with IL-2 (100 U/mL, 20 h) prior to the assay (Figure 4-4B).\(^7\) IR\(^+\) NK cells also tended to lyse target cells and perform all three effector functions (lysis and secretion of both IFN-\(\gamma\) and CCL4) more frequently than IR\(^-\) NK cells, but these comparisons did not achieve statistical significance. When K562 cells were used as the targets, we observed similar trends but with overall lower frequencies of effector function (Figure C-2). We therefore focused our analysis on the interactions between NK cells and antibody-coated P815 target cells.

We stained for a panel of IRs that included NKG2A, KIR2DL1/2/3, and KIR3DL1. It is important to note that in the two donors that were not KIR- and HLA-typed (and thus received the full staining panel), it is possible that a subset of the IR\(^+\) NK cells expressed KIR that did not recognize cognate HLA molecules in the individual. These cells would be unlicensed despite staining positive for IRs. Similarly, in

\(^7\)Microarray analysis of NK cells has shown that the expression of KIR genes is downregulated after 24 h of activation with 100 U/mL IL-2,\(^\text{197}\) but other studies have shown that the surface expression of KIRs does not change significantly after short-term activation with IL-2.\(^\text{198}\) We observed similar frequencies of IR\(^+\) NK cells in the resting and IL-2-activated conditions.
Figure 4-4: **IR**⁺ NK cells secrete cytokines more frequently than **IR**⁻ NK cells do upon interacting with target cells. The cytolytic and secretory activity of single NK cells that formed at least one contact with an antibody-coated P815 target cell were measured and segregated based on whether the NK cell was **IR**⁺ or **IR**⁻. The performance of all three effector functions (All⁺; far right) was also assessed. NK cells were either (A) resting or (B) activated with IL-2 (100 U/mL, 20 h) prior to the assay. Paired data from three donors are shown. The percentage of cells that were positive for each function were corrected for the background frequencies, which were calculated from wells with 0 NK cells and 1–3 target cells (for background lysis) or wells with an NK cell but no targets (for background secretion). Note difference in scale between graphs. *\(P < 0.05\), **\(P < 0.01\), paired t-test.
all donors it is possible that a subset of the IR− NK cells expressed other inhibitory receptors (e.g., the leukocyte Ig-like receptor (LIR)) that were not included in the staining panel. These cells could in theory have been licensed despite staining negative for IRs, although the inhibitory signals delivered by LIR are less dominant than those delivered by KIRs or NKG2A.⁵

4.4.3 Functional differences between IR+ and IR− NK cells responding to target cells are maintained across different durations of contact

The increased functional activity of IR+ NK cells compared with IR− NK cells could have arisen if IR+ NK cells contacted and conjugated with targets more effectively than IR− NK cells did. Indeed, it has been shown that IR− cells form less stable conjugates with K562 target cells than IR+ NK cells do, and that this impaired ability to conjugate with a target cell makes IR− cells less likely to mediate cytolysis.¹⁸³ We therefore sought to determine the extent to which differences in contact with target cells contributed to the differences in secretory activity that we observed. In these assays, we used the murine P815 cell line as target cells. Importantly, human LFA-1 binds murine ICAM-1,¹⁹⁹ so any deficiencies in conjugate formation caused by impaired inside-out signaling to LFA-1 in unlicensed NK cells¹⁸³ should be observable.

From the time-lapse microscopy measurements of individual interactions between NK cells and antibody-coated P815 target cells, we calculated the longest continuous period of time that each NK cell spent in contact with a target cell (Figure 4-5). Both IR+ and IR− NK cells exhibited a broad distribution of contact times. Aggregating data from three donors, the median durations of each NK cell’s longest contact with a target cell were 144 min (resting IR+), 120 min (resting IR−), 128 min (IL-2-activated IR+), and 104 min (IL-2-activated IR−). However, although the IR− NK cells tended to have shorter periods of longest contact, statistical significance was only achieved in one of the pairwise comparisons after correcting for multiple hypothesis testing (Figure 4-5).
Our finding that the IR$^+$ and IR$^-$ NK cells had similar contact time distributions was somewhat unexpected, because a previous study demonstrated that IR$^-$ cells form less stable conjugates with target cells than IR$^+$ NK cells do.$^{183}$ In this study, however, conjugation was measured by flow cytometry. Measurement of conjugation by flow cytometry necessarily subjects pairs of potentially interacting cells to stringent requirements for attachment due to the mechanical perturbation that is involved in sample preparation. In comparison, measurement of contact in the nanowells does not involve any mechanical perturbation, and thus does not distinguish between cells that are weakly or strongly attached. It is possible that unlicensed NK cells with deficiencies in inside-out signaling to LFA-1 are still able to weakly interact with target cells but are not able to strongly attach. Because we imaged at low magnification (6.3$\times$), we could not accurately distinguish between non-productive contacts and contacts that resulted in stable conjugation, the formation of a synapse, and reorganization of the actin cytoskeleton. Future studies could be performed at higher magnification to distinguish between these states of contact. Distinction between these states of contact could provide one explanation for why not all NK cells that arrest upon contact with a target are cytolytically active.$^{97}$

IR$^+$ and IR$^-$ NK cells had a uniformly high likelihood of participating in at least one contact with a target cell over the course of the assay (94% ± 0.2% for resting IR$^+$ cells, 95% ± 1% for resting IR$^-$ cells, 93% ± 3% for IL-2-activated IR$^+$ cells, and 93% ± 2% for IL-2-activated IR$^-$ cells), suggesting that they had similar propensities for initially finding a target. Thus, both IR$^+$ and IR$^-$ NK cells were capable of spending prolonged periods of time in contact with a target cell, but there was considerable variability in the duration of contact within each population.

We next asked how the variability in contact time was related to the two effector functions (secretion of IFN-$\gamma$ or CCL4) that differed between the IR$^+$ and IR$^-$ NK cells (Figures C-3, C-4, C-5).$^8$ The secretion of IFN-$\gamma$ and CCL4 were both strongly correlated with the duration of the longest contact that an NK cell made with a target cell (Figure 4-6). This correlation suggested that in order to make a fair comparison

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$^8$We also assessed the correlation between contact time and cytolysis (Figures C-6 and C-9).
Figure 4-5: Distribution of contact durations between NK cells and target cells, segregated by the expression of IRs on NK cells. Interactions between NK cells (either resting or IL-2-activated, as indicated) and antibody-coated P815 target cells were tracked (8 min/frame) over the course of the 6 h co-incubation in nanowells. The longest continuous period of contact was scored for single NK cells in wells with target cells. Only NK cells that had at least one period of contact with a target cell were included in the analysis. The difference in contact times within each pair of IR+ and IR− populations were compared by the Mann-Whitney test. Comparisons that were significant at the α = 0.05 level after applying the Benjamini-Hochberg method of controlling the false discovery rate (FDR) are indicated (*).
of the target-induced secretory activity of two different cells, it is necessary to also consider and account for how long each of the cells spends in contact with the target. We therefore compared the frequency with which resting IR$^+$ and IR$^-$ NK cells that spent the same length of time in contact with a target secreted IFN-γ or CCL4 (Figures 4-7 and C-7). Both populations secreted IFN-γ and CCL4 more frequently as they spent longer in contact with a target, but despite forming long contacts, IR$^-$ NK cells secreted less frequently$^9$ than IR$^+$ NK cells with matched durations of contact did. These results suggested that although the expression of IRs (and hence licensing) affects the ability of NK cells to form stable conjugates with targets,$^{183}$ even IR$^-$ NK cells that do spend a prolonged period of time in contact with a target are less functionally active than their IR$^+$ counterparts.

4.4.4 Transcriptional profiling of licensed and unlicensed NK cells

The results of the single-cell functional studies indicated that there were underlying differences in the characteristics of IR$^+$ and IR$^-$ NK cells that could not be completely accounted for by differences in their ability to engage target cells. We sought to uncover these differences by using RNA-Seq to generate transcriptional profiles of NK cells with distinct repertoires of IRs and their corresponding HLA ligands.

To investigate licensing in a highly defined subset of NK cells, we focused on comparing KIR3DL1$^+$ and KIR3DL1$^-$ NK cells from donors that did or did not express the cognate HLA ligand (HLA-Bw4) for KIR3DL1. To isolate the effects of licensing through KIR3DL1 as much as possible, we excluded confounding licensing interactions through a combination of donor selection (by excluding donors with certain KIR-HLA pairings) and flow sorting (by excluding NK cells expressing other IRs). This strategy allowed us to compare licensed NK cells (KIR3DL1$^+$ cells from HLA-Bw4 donors) and two sets of unlicensed NK cells (KIR3DL1$^-$ cells from all donors;

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$^9$The median intensity of positive secretion events was similar across the two subsets of NK cells (Figure C-8). This similarity suggests that IR$^+$ and IR$^-$ NK cells differ in their abilities to commence secreting rather than in their ability to produce cytokines once they have committed to secretion.
Figure 4-6: NK cells secrete cytokines with a probability that depends on their longest duration of contact with a target cell. The fraction of NK cells that secreted IFN-γ (top) or CCL4 (bottom) is plotted as a function of the duration of their longest continuous period of contact with antibody-coated P815 target cells. NK cells were either used resting (left) or after activation with IL-2 (right). Trend lines were estimated by LOESS regression; shading represents the 95% confidence interval.
Figure 4-7: IR- NK cells are less likely than IR+ cells to secrete cytokines, even after prolonged contact with a target. The secretory activity of resting NK cells interacting with antibody-coated P815 target cells is plotted as in Figure 4-6 but is segregated based on whether the NK cell was IR+ (green) or IR- (orange). Contact durations were binned due to the low number of events in the IR- population. Trend lines were estimated by LOESS regression; shading represents the 95% confidence interval.
KIR3DL1$^+$ cells from HLA-Bw6/Bw6 donors).

To select donors with suitable characteristics, we screened a cohort of KIR- and HLA-typed healthy donors according to the following criteria:

**All donors**

- Donors who were positive for KIR3DL1 were included, with exceptions as listed below.

- Donors who were positive for the activating receptor KIR3DS1 were excluded,\textsuperscript{10} because although the ligand for KIR3DS1 is not definitively known, there is genetic evidence that KIR3DS1 interacts with HLA-Bw4.\textsuperscript{200}

- Donors who had NK cells with low surface expression of KIR3DL1 as measured by flow cytometry were excluded.\textsuperscript{11} In this cohort, we did not have detailed KIR typing (at the level of individual alleles) for all donors, and so we used this surrogate measure to exclude donors who presumably expressed an allele of KIR3DL1 that results in low surface expression of the receptor ($KIR3DL1^*005$, $*006$, $*007$, $*053$, $*054$). These low-expressing alleles have been shown to abrogate licensing effects.\textsuperscript{187}

- Donors who had NK cells with no surface expression of KIR3DL1 as measured by flow cytometry were excluded. As described above, we used this surrogate measure to exclude donors that presumably were homozygous for $KIR3DL1^*004$, an allele that causes KIR3DL1 to be retained intracellularly.\textsuperscript{201}

- Donors who were HLA-A$^*$03 or HLA-A$^*$11 were excluded, because these HLA molecules are ligands for KIR3DL2.\textsuperscript{202} KIR3DL2 is a framework gene that is present in almost all KIR haplotypes, and thus we could not exclude donors that were positive for this KIR. We note that interactions between KIR3DL2

\textsuperscript{10}These donors were initially included, but sequencing data from these samples were excluded from the final analysis of licensing interactions.

\textsuperscript{11}As with KIR3DS1, these donors were initially included, but sequencing data from these samples were excluded from the final analysis of licensing interactions.
and HLA-A*03 or HLA-A*11 are not thought to mediate strong licensing, and so it may not have been necessary to exclude donors with this KIR-HLA pair.

**Donors expressing HLA-Bw4 (cognate HLA ligand for KIR3DL1)**

- Donors who were homozygous (HLA-Bw4/Bw4) or heterozygous (HLA-Bw4/Bw6) for HLA-Bw4 were included.

**Donors not expressing HLA-Bw4 (cognate HLA ligand for KIR3DL1)**

- Donors who were homozygous (HLA-Bw6/Bw6) for HLA-Bw6 were included.
- Donors who were HLA-A*23, *24, *25, or *32 were excluded, because these HLA molecules are ligands for KIR3DL1.

We flow sorted KIR3DL1+ and KIR3DL1− NK cells (gating to exclude confounding IRs, as described in Section 4.3.2) from three donors who expressed HLA-Bw4 (two HLA-Bw4/Bw4 and one HLA-Bw4/Bw6) and two who did not (HLA-Bw6/Bw6). In each population, we typically obtained $10^3$ – $10^4$ cells. After isolating the RNA and performing RNA-Seq, we compared the expression of KIR3DL1 in the cells that were sorted as being KIR3DL1+ or KIR3DL1−. In each sample, KIR3DL1 was highly expressed in the KIR3DL1+ sorted population compared with the KIR3DL1− sorted population, as expected. In each of these comparisons, KIR3DL1 was in the top 1% of differentially expressed (over expressed in the KIR3DL1+ population) transcripts by GFOLD ranking.

Licensing depends on both the KIR status of the NK cell (KIR3DL1+ or KIR3DL1−) and on the HLA background of the donor (positive or negative for HLA-Bw4, the cognate ligand of KIR3DL1). To identify transcripts whose expression was associated with this interaction, we approached the problem as a multifactorial experiment and used DESeq to fit generalized linear models (GLMs) with a design matrix specifying the KIR status, HLA type, and stimulation condition (with or without antibody-coated P815 target cells) of each sample. Comparison of models with or without the
KIR-HLA interaction term returned two differentially expressed transcripts (FDR < 0.1, Benjamini-Hochberg method): *IL1R2* and *S100A13*. *IL1R2* was expressed predominantly in KIR3DL1− NK cells from HLA-Bw4 donors, while *S100A13* was expressed in KIR3DL1+ NK cells from HLA-Bw4 donors and KIR3DL1− NK cells from HLA-Bw6/Bw6 donors. IL-1R2 is a decoy receptor for IL-1. It has a truncated cytoplasmic domain (rendering it unable to signal) but competes with IL-1R1 (which does propagate signals) for ligands. S100A13 is a member of the S100 family of proteins, which are involved in a variety of cellular processes. S100A13 forms a complex with IL-1α to facilitate the secretion of this cytokine.

The biological significance of the differential expression of proteins related to the IL-1 pathway in NK cells with different KIR-HLA profiles is unclear, and the results of these transcriptional profiles should be validated at the protein level (e.g., by surface staining for IL-1R2) in a larger set of donors. Furthermore, it should be noted that because our analysis included only a small number of donors, it lacked the statistical power necessary to detect a broader range of differentially expressed transcripts. Similar results were obtained from pairwise comparisons across KIR3DL1 status and HLA type. These results are consistent with previous studies indicating that at least some aspects of licensing are regulated by non-transcriptional mechanisms.

In contrast, when we compared NK cells that were treated with different stimulation conditions (unstimulated or incubated with antibody-coated P815 target cells) without considering KIR status or HLA type, we recovered a set of 73 differentially expressed transcripts that were enriched in immune-related gene sets (Gene Set Enrichment Analysis; GSEA), as expected. Overall, the population-level transcriptional profiling highlighted that a large cohort of donors is needed to properly examine differences that are expected to be subtle (as in the differences between licensed and unlicensed NK cells) and to have high donor-to-donor variability. In ongoing work, we are pursuing an alternative approach to investigate potential transcriptional signatures of licensing by applying single-cell RNA-Seq to characterize NK cells with distinct repertoires of IRs from within the same donor (see Chapter 6).
4.5 Conclusions

In this chapter, we described an integrated platform to correlate the expression of IRs on individual NK cells with their functional activity and dynamic properties during interactions with target cells. Using this approach, we demonstrated that functional differences in licensed (IR+) and unlicensed (IR−) NK cells could be detected in the nanowell-based assay. We found that unlicensed NK cells were less likely than licensed NK cells to secrete cytokines, even after prolonged contact with antibody-coated P815 target cells. Thus, potential differences in contact behavior are alone not enough to completely explain the increased ability of licensed NK cells to respond to target cells.
Chapter 5

Cellular Barcodes for Efficiently Profiling Single-Cell Secretory Responses by Microengraving

This chapter is presented as it appeared in Ref. 207, with minor modifications.


*Equal contribution
5.1 Abstract

We present a method that uses fluorescent cellular barcodes to increase the number of unique samples that can be analyzed simultaneously by microengraving, a nanowell array-based technique for quantifying the secretory responses of thousands of single cells in parallel. Using $n$ different fluorescent dyes to generate $2^n$ unique cellular barcodes, we achieved a $2^n$-fold reduction in the number of arrays and quantity of reagents required per sample. The utility of this approach was demonstrated in three applications of interest in clinical and experimental immunology. Using barcoded human peripheral blood mononuclear cells and T cells, we constructed dose-response curves, profiled the secretory behavior of cells treated with mechanistically distinct stimuli, and tracked the secretory behaviors of different lineages of CD4$^+$ T helper cells. In addition to increasing the number of samples analyzed by generating secretory profiles of single cells from multiple populations in a time- and reagent-efficient manner, we expect that cellular barcoding in combination with microengraving will facilitate unique experimental opportunities for quantitatively analyzing interactions among heterogeneous cells isolated in small groups ($\sim$2–5 cells).

5.2 Introduction

Immune cells secrete cytokines to coordinate intercellular communication within the immune network. There is great interest in profiling the secretory activity of immune cells because the cytokines they secrete play a central role in the maintenance of immune homeostasis, the elimination of infectious pathogens, and the induction of allergic and autoimmune responses. The considerable cell-to-cell variability present within populations of immune cells underscores the importance of analytical techniques that enable high-throughput, single-cell secretory measurements.

We previously developed a technique called microengraving that uses dense arrays of subnanoliter wells (nanowells) to quantify the secretion of multiple cytokines from thousands of individual cells in parallel. Cells are isolated in an array of
nanowells, and a glass slide bearing cytokine-specific antibodies is compressed on the array to capture the cytokines secreted by the cells in each well. Single-cell secretory profiles are created by registering the spatial address of each spot on the resulting microarray of secreted proteins back to the corresponding nanowell, and hence the cell(s), that produced the cytokines. Microengraving can be repeatedly performed on the same cells in a nondestructive manner, enabling analytical processes that are not feasible using destructive or end-point single-cell measurements of cytokine production (e.g., intracellular cytokine staining or ELISPOT). These processes include the retrieval of viable cytokine-secreting cells and longitudinal tracking of single-cell secretory profiles.

To date, microengraving has been performed with a throughput of one sample of cells per process. In many cases, however, the analysis of multiple samples in parallel would increase experimental efficiency. One strategy for multiplexing is to use unique combinations of fluorescent dyes to identify distinct groups of cells. This strategy, known as fluorescent cellular barcoding, has been used to increase the throughput of flow cytometry and cell-based assays for drug screening, as well as to track the behavior of specific cells within complex populations. In this chapter, we describe the development and validation of three sets of cellular barcodes that are compatible with microengraving. Application of these cellular barcodes enables the simultaneous analysis of multiple samples of cells by microengraving on a single array of nanowells and thus increases sample throughput, minimizes sample-to-sample technical variability, and reduces both the number of arrays and the quantity of reagents used. Moreover, cellular barcoding opens the door to new applications of microengraving, such as the quantitative analysis of secretory networks governing cell-cell interactions in multicelled wells.
5.3 Materials & Methods

5.3.1 Fabrication of arrays of nanowells

Poly(dimethylsiloxane) (PDMS) (Sylgard 184 Silicone Elastomer Kit; Dow Corning, Midland, MI) arrays of nanowells comprising 50 µm cubic wells (84,672 wells/array) were prepared on 75 × 25 mm² glass slides (Corning, Lowell, MA) following previously reported protocols [126] with minor adaptations. Details can be found in the Supplementary Information (Appendix D.1.1).

5.3.2 Cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) from the whole blood of healthy donors (Research Blood Components, Boston, MA) and were either used fresh or cryopreserved. Before use, cryopreserved PBMCs were thawed, washed with complete media (RPMI-1640 (Mediatech, Manassas, VA) supplemented with 10% heat inactivated fetal bovine serum (FBS; PAA Laboratories, New Bedford, MA), 2 mM l-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mM HEPES (all from Mediatech)), and then rested overnight (37°C, 5% CO₂) in complete media. T cells were isolated from PBMCs by negative selection (EasySep Human T Cell Enrichment Kit; STEMCELL Technologies, Vancouver, BC, Canada) and incubated in complete media until use. Details on T helper (Th) cell polarization can be found in the Supplementary Information (Appendix D.1.2).

5.3.3 Stimulations

Prior to performing the dye-swap experiment, T cells (10⁶ cells/mL) were stimulated in a conical tube for 3 h with 25 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 µM ionomycin (both from Sigma-Aldrich, St. Louis, MO). To evaluate the effect of dose on the functional responses to PMA/ionomycin, T cells (75,000 cells/well) were stimulated in a 96-well flat-bottom plate for 5 h with 10 ng/mL PMA and 0, 0.25,
0.5, or 1 µg/mL ionomycin. To compare diverse stimulation conditions, PBMCs (10^6 cells/well) were incubated in a 96-well U-bottom plate in media only or stimulated for 4 h with PMA/ionomycin (10 ng/mL PMA, 1 µM ionomycin), the Toll-like receptor 4 (TLR4) agonist lipopolysaccharide (LPS-EK, 1 µg/mL; InvivoGen, San Diego, CA), or the TLR7/8 agonist R848 (1 µg/mL; InvivoGen). To test CD4^+ Th cell lineages, Th0-, Th1-, or Th2-polarized cells (10^5 cells/well) were incubated in a 96-well U-bottom plate in media only or stimulated for 4 h with PMA/ionomycin (10 ng/mL PMA, 1 µg/mL ionomycin) as indicated.

5.3.4 Cellular barcoding

Three sets of cellular barcodes were applied as described below. Cells were then washed twice with media and either loaded into a 96-well plate to determine classification accuracy or mixed and loaded onto an array of nanowells for imaging and microengraving.

1. Antibody-based barcoding. Groups of cells were barcoded by the combinatorial application of anti-CD45-quantum dot (QD) 705 (20 nM; Invitrogen) and anti-CD45-QD800 (20 nM; Invitrogen) in media for 15 min at room temperature. Four (2^2) unique barcodes were defined on the basis of QD705 and QD800 staining.

2. Cytosolic barcoding. Groups of cells were barcoded by the combinatorial application of the membrane-permeable dyes carboxyfluorescein diacetate succinimidyl ester (CFSE, 1 µM; Invitrogen, Grand Island, NY) and CellTracker Red (CTR, 2.5 µM; Invitrogen). Four (2^2) unique barcodes were defined on the basis of CFSE and CTR staining. Cells were labeled by directly adding the appropriate combination of dyes to cell suspensions during the last 30 min of stimulation.

3. Streptavidin-based barcoding. Cells were suspended in Hanks Balanced Salt Solution (HBSS) at 10^6 cells/mL in 15 mL conical tubes that were previously blocked with 0.5% polyvinyl alcohol to prevent the adhesion of cells. The
cells were labeled with sulfo-NHS-LC-biotin (0.1 mg/mL; Thermo Scientific, Waltham, MA) for 30 min at 4°C. After one wash, groups of cells were barcoded by the combinatorial application of 40 nM of streptavidin-phycoerythrin (PE)-Cy7 (BioLegend), streptavidin-ITK-QD705 (Invitrogen), and streptavidin-ITK-QD800 (Invitrogen) in HBSS for 15 min at room temperature in a 96-well plate that was preblocked with 10% FBS. Eight (2^3) unique barcodes were defined on the basis of PECy7, QD705, and QD800 staining.

5.3.5 Loading of cells onto arrays of nanowells

Cells originating from different groups were combined into a single suspension (5 × 10^5 cells/mL) after each group had been uniquely barcoded. Then, 300 µL of cell suspension was deposited onto the array. To minimize the chance of cell-cell interactions occurring in the mixed cell suspension, cells were deposited on the array within minutes of being combined together. Cells were allowed to settle by gravity for 5 min before the array was washed gently with media.

5.3.6 Detection of secreted proteins by microengraving

Immediately after labeling and loading the cells, microengraving was performed for 1 h to detect interferon-γ (IFN-γ), macrophage inflammatory protein-1β (MIP-1β), interleukin-2 (IL-2), IL-4, IL-6, and tumor necrosis factor-α (TNF) using previously reported protocols^60,126 with minor adaptations. The specific cytokines analyzed in each experiment are indicated in the text. Additional details can be found in the Supplementary Information (Appendix D.1.3).

5.3.7 Staining for viability and surface marker expression

Where indicated, cells were stained on the arrays with anti-CD8-AlexaFluor647 (2 µg/mL; BioLegend) or anti-CD3-PerCP-eFluor710 (2 test volumes; eBioscience, San Diego, CA). Staining solutions were applied to the arrays for 30 min at room temperature or 4°C and then washed with media. Shortly before imaging, each array
was covered with 200 µL of the viability dye calcein violet (2 µM; Invitrogen). Lifter slips (Electron Microscopy Sciences, Hatfield, PA) were placed on top of the arrays to prevent drying during imaging.

5.3.8 Imaging cytometry

The arrays were imaged using an automated, inverted epifluorescence microscope (Axio Observer, 10×/0.3 objective; Carl Zeiss, Jena, Germany; or Eclipse Ti, 10×/0.45 objective; Nikon Instruments, Tokyo, Japan) with an EM-CCD camera (ImagEM; Hamamatsu Photonics, Hamamatsu, Japan; or iXon3; Andor Technology, South Windsor, CT). A custom-written MATLAB script (Enumerator) was used to analyze the images. This script returned each cell’s nanowell “address” and its intensity in each fluorescent channel (Figure D-1).

5.3.9 Data analysis

Custom-written scripts (MATLAB R2010b; MathWorks, Natick, MA) were used to assign well occupancies and correlate imaging cytometry data with secreted protein data for each well. Only viable cells (calcein violet+) were included in the analysis. Statistical tests were performed in Prism 5 (GraphPad Software, La Jolla, CA). Additional details can be found in the Supplementary Information (Appendix D.1).

5.4 Results & Discussion

5.4.1 Overview of cellular barcoding applied to multiplex single-cell secretory measurements

To implement cellular barcoding in conjunction with microengraving, live cells originating from different populations were labeled with unique combinations of fluorescent dyes, combined, and then loaded onto a single array of nanowells (Figure 5-1). Imaging cytometry was used to determine each cell’s barcode (corresponding to the cell’s
5.4.2 Development and validation of cellular barcodes

We developed and validated three sets of cellular barcodes for use in nanowell-based assays (Figure 5-3). The first set targeted hematopoietic cells (such as immune cells) expressing CD45 on their surface. Four antibody-based barcodes were created by labeling CD45\(^+\) cells with combinations of two different fluorophore-conjugated antibodies against CD45 (Figure 5-3A). This strategy of barcoding is extendable to other surface-expressed markers common to all populations of cells of interest but cannot be applied if cells express surface proteins heterogeneously or if suitable antibodies are unavailable. We therefore established a second set of cellular barcodes to label cells with four combinations of two fluorescent, cytosolic dyes (CFSE and CTR) (Figure 5-3B). Unlike antibody-based barcodes, cytosolic barcodes are suitable for general use with all cell types and are durably retained in cells for hours to days. These features make cytosolic barcodes useful for experiments that involve mixed cell types or that require deconvolution of populations after prolonged culture. Finally, to extend the depth of barcoding, we created streptavidin-based barcodes by biotinylating cells and then labeling them with eight combinations of three different fluorophore-conjugated streptavidins (streptavidin-PE-Cy7, streptavidin-QD705, and streptavidin-QD800) (Figure 5-3C). The high efficiency of cellular labeling with biotin-streptavidin and the large spectral selection of commercially available streptavidin-fluorophore conjugates make this approach useful when a high depth of barcoding is needed (e.g., parallel analysis of disaggregated tissue biopsies).

All three sets of barcodes produced uniform, unambiguous cellular staining (Figure 5-3) and a reproducibly high accuracy of objective classification (Figure 5-4 and Table D.1; calculations described in Appendix D.1.4). We note that factors such as the resolution of the microscope, size of the cells, and density of the cells in the nanowells can influence the accuracy of classification; thus, it is important to measure the
Figure 5-1: Schematic for using cellular barcodes to increase the throughput of secretory measurements from single cells. (1) Distinct groups of cells (e.g., from different treatment conditions) are labeled with unique combinations of fluorescent dyes (barcodes). (2) The cells are combined and loaded onto the array of nanowells. (3) Viability and surface marker expression (labeled on-chip), as well as the barcodes of each cell, are determined by imaging cytometry. Microengraving is performed to measure the factors secreted by the cells in each well. (4) Barcodes are deconvolved during image analysis to identify each cell’s group of origin. Data from imaging cytometry and microengraving are matched on a per-well basis.
Figure 5-2: **Matched imaging cytometry and microengraving.** Representative composite micrographs of imaging cytometry (left) and corresponding microarray of secreted proteins (right) from a 7 × 7 block of nanowells containing barcoded cells. In this example, calcein violet was used as the viability dye, CFSE was used as barcode dye 1, and CTR was used as barcode dye 2.

As with any perturbation, the application of barcodes could potentially affect the biology of the cell. For example, functionalization of the cell surface for labeling might affect the cell’s ability to respond to autocrine cues. It was therefore necessary to assess whether barcoding perturbed the cellular functions measured over the time scale of interest here (hours). Accordingly, we performed a dye-swap experiment to validate that barcoding did not affect the short-term secretory responses of the cells. Primary human T cells were stimulated with PMA and ionomycin, divided into aliquots, barcoded, and loaded together onto an array of nanowells. Microengraving was then performed to quantify the secretion of IFN-γ, MIP-1β, and IL-2 from the cells in each barcoded group. The percentage of single cells that secreted each cytokine was uniform across all barcoded groups within a given set of barcodes (Figure 5-5). The interbarcode coefficients of variation (CVs) were 3–14% (IFN-γ), 3–14% (MIP-1β), and 2–12% (IL-2). These CVs are comparable to the interassay variability
Figure 5-3: **Barcoded T cells.** Cells were labeled with (A) antibody-based barcodes, (B) cytosolic barcodes, or (C) streptavidin-based barcodes. The “Negative” population denotes cells that were not labeled. AFU, arbitrary fluorescence units (logicle transformation).

of other single-cell assays measuring the production of cytokines, including intracellular cytokine staining\(^{217,218}\) and ELISPOT.\(^{219}\) Furthermore, the median fluorescence intensities (MFIs) for positive secretion events were similar across barcoded groups (Figure D-2). In each set of barcodes, one group of cells was not labeled until after microengraving and thus served as an internal, unperturbed control. The secretory response of this group was not significantly different from the responses of the other barcoded groups, further indicating that the application of the barcodes did not affect the short-term secretory biology of the cells. We therefore proceeded to use cellular barcoding in three applications relevant to experimental and clinical immunology.

### 5.4.3 Application 1: Efficient construction of dose-response curves

Many immunological assays rely on stimulating cells with a chemical stimulant or an antigen and then measuring the resulting secretion of cytokines. In these assays, it is often beneficial to test a range of doses to determine the optimal concentration
Figure 5-4: **Classification accuracy.** Classification accuracy of cells labeled with (A) antibody-based barcodes, (B) cytosolic barcodes, or (C) streptavidin-based barcodes.

Figure 5-5: **Dye-rotation experiments to validate that the application of barcoding dyes does not affect the short-term secretory profiles of cells.** Secretory responses were compared among uniformly stimulated T cells that received different (A) antibody-based barcodes, (B) cytosolic barcodes, or (C) streptavidin-based barcodes. For each analyte, the frequency of secretion observed from cells with different barcodes was normalized to the unlabeled group of cells (Barcode 1; dark grey). The mean and range of three replicates are shown. For each analyte and barcode set, there was no significant difference \((P > 0.05)\) in normalized secretion among the different barcodes (one-way analysis of variance (ANOVA)).
of the stimulating reagent. We used cytosolic barcodes to efficiently measure, on a single array of nanowells, the secretory response of human T cells activated with 10 ng/mL PMA in combination with four doses of ionomycin (0, 0.25, 0.5, and 1 µg/mL). After microengraving and deconvolution of barcoded single cells, we observed a dose-dependent increase in secretion (Figure 5-6, top panel). By staining for CD8 (a surface marker that distinguishes subsets of T cells), we identified subset-specific differences in secretion (Figure 5-6, bottom panel). In response to increasing doses of ionomycin, the percentage of CD8– T cells secreting IL-2 increased sharply, but the percentage of CD8+ T cells secreting IL-2 remained at basal levels. In contrast, the percentage of CD8+ T cells secreting MIP-1β increased strongly in response to increasing doses of ionomycin, but the percentage of CD8– T cells secreting MIP-1β remained close to basal levels. These observations are consistent with previous studies. Together, these results demonstrate that cellular barcoding allows dose-response curves to be efficiently constructed from a single microengraving process.

5.4.4 Application 2: Profiles of secretory responses induced by diverse stimuli

Clinical studies are currently trying to establish robust methods to monitor human immune responses. One approach is to test how immune cells respond to diverse stimuli that trigger distinct signaling pathways. To test this scenario, we applied cytosolic barcodes to PBMCs treated with four different stimulation conditions and measured their secretory responses by microengraving. We observed secretion profiles consistent with the class of stimuli applied (Figure 5-7, top panel). Unstimulated cells had low numbers of secreting single cells for all cytokines. Treatment with PMA and ionomycin stimulated the secretion of IL-2 and TNF, whereas treatment with TLR agonists LPS or R848 induced the secretion of IL-6 and TNF. By staining for the T cell-specific surface marker CD3, we identified CD3+ T cells as the dominant population responding to stimulation with PMA and ionomycin (Figure 5-7, bottom panel). In contrast, the majority of secretory responses to both TLR stimuli
Figure 5-6: **Application of cellular barcodes to construct dose-response curves.** Barcoded T cells were stimulated with 0, 0.25, 0.5, or 1 µg/mL ionomycin and 10 ng/mL PMA. Microengraving was used to measure single-cell secretory responses, and surface phenotype was distinguished by on-chip labeling with anti-CD8. The numbers of single cells analyzed are indicated in parentheses.
Figure 5-7: Application of cellular barcodes to measure the percentage of single PBMCs secreting cytokines after treatment with mechanistically distinct stimuli. Barcoded PBMCs were stimulated with PMA/ionomycin (P/I), R848, LPS, or left unstimulated (–). Microengraving was used to measure single-cell secretory responses, and surface phenotype was distinguished by on-chip labeling with anti-CD3. The numbers of single cells analyzed are indicated in parentheses.

came from CD3− cells. These findings are consistent with existing knowledge on the secretory responses induced by each of these stimuli\textsuperscript{224,225} and show that cellular barcoding can be used to multiplex the analysis of diverse stimulatory conditions for applications of microengraving in immune monitoring, such as the rapid evaluation of immunological responses to disease states or vaccination.
5.4.5 Application 3: Profiles of lineage-dependent secretory responses

Different lineages of CD4\(^+\) T helper (Th) cells have distinct transcription factors and secretory profiles\(^{226}\) but cannot be distinguished by their surface markers. To measure the secretory profiles of multiple lineages on a single array of nanowells, we used cytosolic barcodes to label PMA/ionomycin-stimulated Th cells that had been cultured under Th0-, Th1-, or Th2-polarizing conditions, which each promote distinct secretory patterns. Th1 cells can secrete IFN-\(\gamma\) and IL-2, Th2 cells can secrete IL-4 and IL-2, and Th0 cells can secrete both Th1 and Th2 cytokines,\(^{226-229}\) although \textit{in vitro} polarization does not produce 100% conversion.\(^{230}\) After microengraving and deconvolution of barcoded single cells, we found that the percentages of single cells from each lineage that secreted IL-2, IFN-\(\gamma\), or IL-4 (Figure 5-8A), as well as the rates at which secretion-positive cells from each lineage secreted each cytokine (Figure 5-8B), were both consistent with the expected lineage-specific secretory patterns. Together, these results show that cellular barcoding in combination with microengraving enables efficient tracking of the identities of T cells that have distinct secretory profiles but indistinguishable sets of surface-expressed markers.

5.5 Conclusions

Here, we have demonstrated three sets of cellular barcodes that can be used to increase the throughput of single-cell secretory measurements by \(2^n\)-fold, where \(n\) is the number of dyes used to generate the barcodes. Although we focused on single cells, the platform is also well suited for measuring the secretory profiles from small groups of cells (2–5 cells/nanowell) with precisely defined demographics. We anticipate that the combination of cellular barcoding and microengraving will enable the quantitative analysis of cell-cell interactions with a resolution that has not been possible using traditional experimental systems and will yield novel insights into the mechanisms governing the behavior of complex cellular systems.
Figure 5-8: Application of cellular barcodes to profile lineage-dependent secretory responses from CD4\(^+\) Th cells. Single-cell secretory responses from barcoded CD4\(^+\) cells biased to Th0, Th1, or Th2 and then stimulated with PMA/ionomycin (P/I) or left unstimulated (–). (A) Percentage of secreting single cells from each population of Th cells. (B) Intensities of secretion from single Th cells. Only positive secretion events are shown. Red lines indicate the mean and the standard error of the mean. *\(P < 0.05\), ***\(P < 0.0001\), Kruskal-Wallis test followed by Dunn’s post-test comparing the three groups of Th cells that were stimulated with PMA/ionomycin. The cells were labeled with cytosolic barcodes in this experiment.
Chapter 6

Summary & Future Directions

In this thesis, we introduced a set of tools to study cellular interactions at the single-cell level. We applied these tools to measure the cellular dynamics (e.g., motility, contact time, contact history) of interactions between 100’s – 1000’s of individual NK cells and target cells. We then correlated these dynamic properties with the surface-marker phenotype of each NK cell and the functional outcomes (e.g., cytolysis, secretion of cytokines) that resulted from each interaction. From these integrated single-cell profiles of dynamics, function, and surface-marker phenotype, we uncovered new properties by which NK cells perform immune surveillance, attack target cells, regulate their expression of surface markers, and drive downstream immune responses. Although here we focused on the interactions between NK cells and target cells, this platform can be adapted to study a wide range of intercellular interactions of interest.

The nanowell-based platform facilitated the analysis of both individual cells and multicellular systems. In addition to identifying associations between distinct dynamic, functional, and phenotypic properties in single NK cells, the analysis of large numbers of nanowells containing different combinations of NK cells and target cells enabled the identification of multicellular processes that occur either independently (as we observed with cytolytic activity) or in a manner that is more coordinated than expected by chance alone (as we observed with the shedding of CD16 from the surface of NK cells). In contrast, measurements of isolated cells or of bulk mixtures of cells are not amenable to this type of cooperativity analysis. The strategies for fluorescent
cellular barcoding that were initially applied here to increase the throughput of single-cell secretory measurements can be extended to further define the cooperative—or independent—behaviors of cells in multicellular systems.

The results presented here offer many possibilities for future extensions:

**Alternative target cells**

In the experiments presented here, we used K562 cells and P815 cells (coated with antibodies to induce ADCC) as targets for NK cells. Alternative target cell lines, or even primary cells (e.g., virally infected cells), could be used to provide distinct stimuli to NK cells. To provide inhibitory signals through specific KIR, MHC Class I deficient target cell lines (722.221) could be transfected with different HLA Class I allotypes. These cells would be useful for characterizing the inhibition experienced by NK cells expressing distinct repertoires of IRs. In addition, a variety of different target cells could be used to further study ADCC, other CD16-mediated effector functions, and the shedding of CD16. In the context of cancer therapy, B cells (Raji) coated with rituximab would provide a clinically relevant model of an antibody-coated target cell, as would HER2+ breast cancer cells coated with trastuzumab. In the context of HIV, HIV-infected CD4+ T cells coated with serum from an HIV+ patient would provide a clinically relevant model of an antibody-coated target cell. The use of these model cell lines would likely influence the functional activity observed in the assay, as it has been shown that the secretion of cytokines from NK cells mixed with antibody-coated target cells is correlated with the surface density of antibody on the target. Furthermore, these target cells would present other relevant ligands (e.g., HLA molecules).

**Combinations of target cells**

In a diseased tissue (e.g., tumor or site of infection), NK cells may encounter target cells coated with antibodies that activate NK cells through CD16 as well as target cells that express stress-induced ligands that activate NK cells through NKG2D or other receptors. To simulate these heterogeneous mixtures, different types of target
cells could be co-loaded into the nanowells. This system would facilitate investigation of how the activation-induced downregulation of these receptors affects history-dependent cytolytic responses of NK cells as they serially encounter target cells that present ligands for either CD16 or NKG2D (or other receptors). At the end-point of the assay, on-chip fixation, permeabilization, and staining for surface markers as well as cytolytic molecules such as perforin could be used to infer which NK cells failed to kill late-encountered targets due to receptor downregulation and which failed to kill due to depletion of cytolytic granules.

**CD16 shedding behavior: Reporters and perturbations**

To monitor the activity of the proteases that are responsible for the shedding of CD16, FRET-based reporter peptides could be spiked into the media and introduced to the array of nanowells.\textsuperscript{232,233} To perturb the activity of the proteases, specific or broad-spectrum protease inhibitors could be used.\textsuperscript{152–157} Together, these approaches would further define the kinetics of CD16 shedding from NK cells upon contact with target cells, as well as the functional consequences of CD16 shedding.

**Monitor CD16 shedding in live tissues**

Dynamic in situ cytometry (DISC) has been used to monitor the shedding of CD62L from T cells as they encounter and are activated by APCs in living tissues.\textsuperscript{84} DISC could be applied in a similar fashion to monitor the shedding of CD16 from NK cells as they interact with target cells. It should be noted, however, that studies using murine neutrophils have shown that murine CD16 is not appreciably shed upon cellular activation (although CD62L is),\textsuperscript{234} which would complicate the *in vivo* analysis of CD16 shedding. This difference in CD16 shedding in humans compared with mice also raises interesting and clinically important questions about how to interpret data from mouse models testing the efficacy of antibody therapeutics that induce ADCC.

**Single-cell studies of the functional and dynamic properties of NK cells licensed through specific KIR-HLA interactions**

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The approach from Chapter 4 could be extended to investigate the functional properties of NK cells that have been licensed through a specific receptor (instead of a collection of different IRs). For these experiments, donors with specific combinations of KIR and HLA would be selected (e.g., donors positive for KIR3DL1 and either HLA-Bw4 or HLA-Bw6/Bw6). The functional properties of KIR3DL1+ cells from HLA-Bw4 donors would be compared with those from HLA-Bw6/Bw6 donors (and with KIR3DL1− cells from either set of donors). We would expect the first set of cells to be licensed and more functionally active than the latter two sets. To obtain the cells for the nanowell assay, cells would be flow sorted by gating on CD56dim NKG2A− other KIR− NK cells. After the conclusion of the nanowell assay (in which cytolysis, secretion, motility, contact, etc. would be measured), on-chip staining and imaging cytometry would be performed to identify which NK cells were positive for the specific KIR of interest (e.g., KIR3DL1). Although this approach would be useful to investigate functional properties of NK cells licensed through specific inhibitory interactions, it would be technically challenging due to the low number of cells that would be sorted and used on each array of nanowells.

Apply the nanowell assays to investigate the revocable license model

According to the “revocable license” model, licensed NK cells have their license “revoked” after an activating encounter (by rearrangement of activating receptors on the cell surface); the license can be reinstated by an inhibitory (licensing) interaction. Thus, we would hypothesize that cells that receive an inhibitory signal (via an anti-NKG2A or anti-KIR antibody) after an initial encounter with a target will be “relicensed” and more functionally responsive upon a subsequent encounter than cells that do not receive an inhibitory signal between encounters. To test this hypothesis, we would flow sort CD56dim NK cells, perform the nanowell assay (monitoring cytolysis, secretion, motility, contact, etc.) while providing inhibitory signals by including antibody-coated beads in the nanowells or by uniformly coating the surface of the nanowells with the antibodies against the inhibitory receptors, and then stain on-chip at the end-point of the assay for the inhibitory receptor that was triggered.
by the antibody (e.g., NKG2A or KIR). We would expect that cells that expressed the receptor of interest and received an inhibitory signal between interactions would be more functionally active upon subsequent encounters with target cells than those that did not receive the inhibitory signal (and hence remained in the “revoked” state).

**Investigate the transcriptional profiles of single NK cells with distinct repertoires of inhibitory receptors**

In ongoing work, we are using single-cell RNA-Seq to investigate the transcriptional profiles of individual NK cells with distinct repertoires of inhibitory receptors. Single-cell RNA-Seq has been used to characterize the population structure of immune cells and to define subsets of cells based on combinations of expressed transcripts.\(^{68,69}\) Due to the large amount of heterogeneity present within the NK cell population—even after gating on NK cells defined by the expression of several surface markers—we expect that this approach will enable the identification of new signatures and subsets.

In addition, RNA-Seq could be extended to profile individual NK cells or small groups of NK cells retrieved from the nanowells by micromanipulation at the conclusion of the functional assays. The use of the murine P815 target cells is amenable to distinguishing transcripts from NK cells and target cells.
Appendix A

Supplementary Information for Chapter 2

A.1 Supplementary Methods

A.1.1 Fabrication of arrays of nanowells

Arrays of nanowells containing either 30 µm (248,832 wells/array) or 50 µm (84,672 wells/array) cubic wells were prepared on 75 × 25 mm² glass slides (Corning) by injecting a silicone elastomer (poly(dimethylsiloxane) (PDMS; Dow Corning), 10:1 ratio of base:catalyst) into a mold containing a microfabricated silicon master. PDMS was cured at 80°C for 4 h and then released from the mold. Shortly before depositing cells onto the array, the arrays were treated with an oxygen plasma (Harrick PDC-32G) for 30 s to sterilize the array and render the PDMS hydrophilic. Arrays were stored in phosphate-buffered saline (PBS) until use, and were washed with complete media prior to cell loading.

A.1.2 Microengraving to detect secreted proteins

Capture antibodies against MIP-1β (R&D Systems), IFN-γ (Mabtech), and human immunoglobulin G (hIgG) (ZyMax; Invitrogen) (10 µg/mL each in borate buffer; pH 9) were coated onto poly(L-lysine)-coated glass microscope slides for 1 h at room
temperature. Slides were then blocked in 1.5% bovine serum albumin (BSA; EMD
Chemicals) / PBS-TWEEN20 (0.05%; Sigma-Aldrich) (PBST) for 30 min, washed
once in PBS, dipped in water, and spun dry. Prior to microengraving, the cell-
loaded arrays of nanowells were rinsed with serum-free media containing hIgG (34.5
ng/mL; Athens Research & Technology) to provide a positive background signal in
every well and facilitate the registration of the features during image analysis of the
captured protein microarrays. Excess media was aspirated and the capture antibody-
coated glass slides were placed face down on top of the cell-loaded arrays (Figure
2-6). Compression was applied using a microarray hybridization chamber (Agilent).
The clamped arrays were incubated for 1 h (30 µm wells) or 2 h (50 µm wells) to
allow the capture of secreted proteins onto the antibody-coated glass slide. The re-
sulting microarrays of secreted proteins were then separated from the PDMS array,
washed in PBS, blocked with 1.5% BSA-PBST, and hybridized (45 min, room temper-
ature) with the following detection antibodies: biotinylated anti-MIP-1β, anti-IFN-γ-
AlexaFluor555, and anti-hIgG-AlexaFluor700 (1 µg/mL each in 0.1% BSA-PBST, all
from the same manufacturers as the corresponding capture antibodies). Arrays were
washed in PBS and PBST and then hybridized an additional 30 min at room tem-
perature with streptavidin-AlexaFluor647 (1 µg/mL; Invitrogen). After a final series
of washes in PBS, PBST, and water, the protein microarrays were dried and im-
gaged with 5-µm resolution using a commercial microarray scanner (GenePix 4200AL,
Molecular Devices).

The microarray of secreted proteins was analyzed using commercial image process-
ing software (GenePix Pro 6, Molecular Devices). The median fluorescence intensity
(MFI) in each channel was calculated for each spot in the array to determine the
relative intensity of secretion of the cells in the corresponding nanowell. Data were
filtered to exclude spots with saturated pixels or high coefficients of variation (>70–
90). Spots with a high signal-to-noise ratio (>3–5), low relative local background,
and MFI > [global background + 2 standard deviations] were marked as positive
spots.
A.2 Supplementary Figures

Figure A-1: Flow cytometry gating scheme for NK cells. Numbers indicate percentages within each gate. NK cells were defined as live CD3−CD14−CD19− lymphocytes expressing CD56 and/or CD16 (i.e., CD56+CD16− (CD56bright NK cells), CD56+CD16+ (CD56dim NK cells) or CD56−CD16+ (CD56neg NK cells)).

Figure A-2: Surface expression of NKG2D ligands on K562 target cells. Black lines represent the K562 cells stained with the indicated antibody; grey shaded areas show the respective isotype controls.
Figure A-3: Distribution of NK cells and K562 target cells in arrayed nanowells. Bar graph of the mean and standard deviation (SD) of the numbers of nanowells on each array that contained the indicated combinations of NK cells and target cells. Results were compiled from eight arrays containing 30 µm cubic nanowells. Each 75 × 25 mm² array contained 248,832 wells. In all experiments, to minimize any potential edge effects, only wells located in the center of the array were considered (147,456 wells). Further filtering was performed to exclude wells with dead cells at the t = 0 h time-point and wells in which the cell occupancy changed between the images acquired at t = 0 h and t = 4 h.
Figure A-4: **Phenotypes of NK cells after 44 h stimulation with IL-2.** NKp46, NKG2D, CD69, and intracellular perforin were measured by flow cytometry for freshly isolated NK cells (Baseline) or for NK cells stimulated for 44 h in media or in 50 U/ml IL-2. Bar graphs show mean and SD for three donors per condition.
Appendix B

Supplementary Information for Chapter 3

B.1 Supplementary Methods

B.1.1 Antibodies for flow and imaging cytometry

Cells were stained for flow or imaging cytometry using the antibodies or isotype controls (ITCs) listed in Table B.1 and described in the text.

Table B.1: Antibodies for flow and imaging cytometry

<table>
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<th>Surface Marker</th>
<th>Fluorophore</th>
<th>Clone</th>
<th>Supplier</th>
<th>Use</th>
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B.2 Supplementary Figures

Figure B-1: **Expression of CD16 on resting CD56^{dim} NK cells after stimulation with target cells in bulk.** Resting PBMCs (i.e., not activated with IL-2) were incubated with (A) antibody-coated P815 target cells or (B) K562 target cells for 0, 0.5, 1, 2, or 4 h and then stained, fixed, and acquired by flow cytometry. CD56^{dim} NK cells were gated as CD3^{-}CD14^{-}CD19^{-}CD56^{dim} lymphocytes.
Figure B-2: NK cells shed CD16 with a probability that depends on their cumulative duration of contact with a target cell and their activation state. The fraction of NK cells that retained expression of CD16 is plotted as a function of their cumulative duration of contact with (A) antibody-coated P815 target cells or (B) K562 target cells. NK cells were either IL-2-activated (orange, combined data from three donors) or resting (green, data from one donor, who was also included in the IL-2-activated data set). Trend lines were estimated by LOESS regression; shading represents the 95% confidence interval.

Figure B-3: NK cells act independently when lysing antibody-coated P815 target cells in nanowells. Wells containing the indicated number of IL-2-activated NK cells and 1–3 antibody-coated P815 target cells were included in the analysis. The predicted fractions of wells with at least one dead target were calculated as described in Ref. 99. Predicted fractions were then compared with the experimentally observed fractions. Data from three donors are shown. The actual (observed) fractions did not differ significantly (at the $\alpha = 0.05$ level) from the predicted fractions according to Pearson’s chi-squared test (regardless of corrections for multiple comparisons).
Figure B-4: Not all NK cells that shed CD16 lyse target cells. The frequency of IL-2-activated NK cells that actively shed CD16 (calculated as described in the text) in response to engaging an (A) antibody-coated P815 or (B) K562 target cell was compared with the frequency with which these cells exhibited cytolytic activity. Paired data from three donors are shown. *$P < .05$, paired t-test.
Appendix C

Supplementary Information for Chapter 4

C.1 Supplementary Methods

C.1.1 Antibodies for flow and imaging cytometry

Cells were stained for flow or imaging cytometry using the antibodies listed in Table C.1 and described in the text.

C.2 Supplementary Figures
Table C.1: Antibodies for flow and imaging cytometry

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<td>Beckman Coulter</td>
<td>Flow cytometry</td>
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Figure C-1: Chilling the array of nanowells improves the retention of NK cells. Percentage of single cells retained in nanowells between time-lapse imaging (or, for PBMC control, mock perturbation by sealing with a glass slide) and analysis of surface marker staining by imaging cytometry. For the two NK cell data sets, the array was chilled on ice for the indicated period of time and centrifuged (340 g, 1 min) prior to removing the glass slide that sealed the array during the time-lapse imaging. Cells were then stained on the array for surface markers and imaged again. For the PBMC control, all manipulations were performed at room temperature. Bars indicate median and standard deviation.
Figure C-2: **Functional activity of IR⁺ and IR⁻ NK cells against K562 target cells.** The cytolytic and secretory activity of single NK cells that formed at least one contact with a K562 target cell were measured and segregated based on whether the NK cell was IR⁺ or IR⁻. NK cells were either (A) resting or (B) activated with IL-2 (100 U/mL, 20 h) prior to the assay. Paired data from three donors are shown. The percentage of cells that were positive for each function were corrected for the background frequencies, which were calculated from wells with 0 NK cells and 1–3 target cells (for background lysis) or wells with an NK cell but no targets (for background secretion). Note difference in scale between graphs. *P < .05, paired t-test.
Figure C-3: Distribution of secretion intensities as a function of contact time parameters. Each point represents a well containing a single NK cell that interacted with a target cell with the contact time properties that are indicated at the bottom of each column. The black line indicates the threshold for positive events. The data shown here are from Donor 1 (resting NK cells; antibody-coated P815 target cells).
Figure C-4: **Frequency of secretion as a function of contact time parameters.** The frequencies of single NK cells secreting CCL4 (top row) or IFN-γ (middle row) are plotted as a function of the contact time properties that are indicated at the bottom of each column. The overall frequency of events is shown in the bottom row. The data shown here are from Donor 1 (resting NK cells; antibody-coated P815 target cells).
Figure C-5: Median intensity of positive secretion events as a function of contact time parameters. The background-corrected median fluorescence intensity of positive secretion events from single NK cells secreting CCL4 (top row) or IFN-γ (middle row) is plotted as a function of the contact time properties that are indicated at the bottom of each column. The overall frequency of events is shown in the bottom row. The data shown here are from Donor 1 (resting NK cells; antibody-coated P815 target cells).
Figure C-6: **Frequency of cytolysis as a function of contact time parameters.** The frequency of single NK cells that lysed a target cell is plotted as a function of the contact time properties that are indicated at the bottom of each column. The overall frequency of events is shown in the bottom row. The data shown here are from Donor 1 (resting NK cells; antibody-coated P815 target cells).
Figure C-7: **Frequency of secretion as a function of contact time parameters, segregated by the expression of IRs on NK cells.** The frequencies of single IR+ (green) and IR− (red) NK cells secreting CCL4 (top row) or IFN-γ (middle row) are plotted as a function of the contact time properties that are indicated at the bottom of each column. The overall frequency of events is shown in the bottom row. The data shown here are from Donor 1 (resting NK cells; antibody-coated P815 target cells).
Figure C-8: Median intensity of positive secretion events as a function of contact time parameters, segregated by the expression of IRs on NK cells. The background-corrected median fluorescence intensity of positive secretion events from single IR$^+$ (green) and IR$^-$ (red) NK cells secreting CCL4 (top row) or IFN-γ (middle row) is plotted as a function of the contact time properties that are indicated at the bottom of each column. The overall frequency of events is shown in the bottom row. The data shown here are from Donor 1 (resting NK cells; antibody-coated P815 target cells).
Figure C-9: **Frequency of cytolysis as a function of contact time parameters, segregated by the expression of IRs on NK cells.** The frequency of single IR\(^+\) (green) and IR\(^-\) (red) NK cells that lysed a target cell is plotted as a function of the contact time properties that are indicated at the bottom of each column. The overall frequency of events is shown in the bottom row. The data shown here are from Donor 1 (resting NK cells; antibody-coated P815 target cells).
Appendix D

Supplementary Information for Chapter 6

D.1 Supplementary Methods

D.1.1 Fabrication of arrays of nanowells

Arrays of nanowells comprising 50 µm cubic wells (84,672 wells/array) were prepared on 75 × 25 mm² glass slides (Corning, Lowell, MA) following previously reported protocols with minor adaptations. To fabricate the arrays, the silicone elastomer poly(dimethylsiloxane) (PDMS) (Sylgard 184 Silicone Elastomer Kit; Dow Corning, Midland, MI) was mixed at a 10:1 ratio of base:catalyst, degassed under a vacuum at room temperature for 1 h, and then injected into a mold containing a microfabricated silicon master. The PDMS was cured at 80°C for 4 h and subsequently released from the mold to produce a glass slide-backed array of nanowells.

Shortly before use, the arrays of nanowells were treated with oxygen plasma (Plasma Cleaner PDC-001; Harrick Plasma, Ithaca, NY) for 2 min to sterilize the array and render the PDMS hydrophilic. Following plasma treatment, the arrays were stored in phosphate-buffered saline (PBS) and then washed and blocked with serum-containing media prior to depositing cells onto the array.
D.1.2 T helper (Th) cell biasing

Naïve CD4⁺ T cells were isolated from fresh PBMCs by negative selection (EasySep Human Naïve CD4⁺ T Cell Enrichment Kit; STEMCELL Technologies). Purity was routinely >95%. Cells were plated at 50,000 cells per well in 96-well U-bottom plates and activated with anti-CD3/28 Dynabeads (Life Technologies, Carlsbad, CA). Th0, Th1, and Th2 cultures were maintained in Xvivo20 (Lonza, Walkersville, MD) and activated with a 2:1 bead:cell ratio. Biasing conditions for each subset were as follows:

- **Th0**: Unsupplemented

- **Th1**: 20 ng/mL IL-2 (Peprotech, Rock Hill, NJ), 10 ng/mL IL-12 (R&D Systems, Minneapolis, MN), and 10 µg/mL anti-IL-4 (BD Biosciences, Franklin Lakes, NJ)

- **Th2**: 20 ng/mL IL-2 (Peprotech), 20 ng/mL IL-4 (R&D Systems), 10 µg/mL anti-IFN-γ (BD Biosciences), and 10 µg/mL anti-IL-12 (BD Biosciences).

On day 3, cells were counted, split to a concentration of 5 × 10⁶ cells/mL, and re-fed with fresh media. On day 5, the beads were magnetically removed, and the cells were re-fed and split. Cells were left resting for 2 days before being restimulated for another 5 days as described above. After resting for another 2 days, cells were used for functional assays.

D.1.3 Detection of secreted proteins by microengraving

Microengraving was performed using previously reported protocols with minor adaptations. Poly(L-lysine)-coated glass microscope slides were coated with a capture antibody against human IgG (ZyMax; Invitrogen) and a set of 3 of the following capture antibodies, as detailed in the text: anti-IFN-γ (Mabtech, Mariemont, OH), anti-MIP-1β (R&D Systems), anti-IL-2 (R&D Systems), anti-IL-6 (BD Biosciences), anti-TNF-α (BioLegend, San Diego, CA), or anti-IL-4 (BioLegend). The capture antibodies were diluted in borate buffer (pH 9) to a concentration of 10 µg/mL.
for each antibody immediately prior to being applied to the glass slide.\textsuperscript{236} Coating was performed at room temperature for 1 h or at 4°C overnight. Slides were then blocked in 1.5% bovine serum albumin (BSA; EMD Chemicals, Gibbstown, NJ) / PBS-TWEEN20 (0.05%; Sigma-Aldrich) (PBST) or in non-fat milk (3% w/v in PBST) for 30 min, washed once in PBS, dipped in water, and spun or blotted to remove excess fluid.

Immediately prior to microengraving, the cell-loaded arrays of nanowells were rinsed with FBS-free media with 0.01% human serum (containing IgG) to provide a positive background signal in every well. This uniform background signal facilitated the registration of the array during image analysis of the captured protein microarrays. Capture antibody-coated glass slides were placed face-down on top of the cell-loaded arrays, and compression was applied using a microarray hybridization chamber (Agilent, Santa Clara, CA). The clamped arrays were returned to the incubator for 1 h to allow the capture of secreted proteins onto the antibody-coated glass slide. The resulting protein microarrays of secreted products were then separated from the PDMS array, washed in PBS, blocked with 1.5% BSA-PBST or 3% milk, and hybridized (45 min, room temperature) with detection antibodies against the analytes of interest. Solutions of detection antibodies were prepared at 1 µg/mL for each antibody in 0.1% BSA-PBST. The following detection antibodies were used: anti-hIgG-AlexaFluor700, anti-IFN-γ-AlexaFluor555, biotinylated anti-MIP-1β (in combination with streptavidin-AlexaFluor647 (1 µg/mL; Invitrogen) applied during an additional 30 min hybridization step), anti-IL-2-AlexaFluor594, anti-IL-6-AlexaFluor555, anti-TNF-α-AlexaFluor488, and anti-IL-4-AlexaFluor647 (all from the same manufacturers as the paired capture antibodies listed above).

The resulting microarrays of secreted proteins were imaged with 5-µm resolution using a commercial microarray scanner (GenePix 4200AL; Molecular Devices, Sunnyvale, CA). The microarrays were analyzed using commercial image processing software (GenePix Pro 6, Molecular Devices). The median fluorescence intensity (MFI) in each channel was calculated for each spot on the array to determine the relative intensity of secretion from the cells in the corresponding nanowell. Data were
filtered to exclude spots with saturated pixels or high coefficients of variation (>100). Spots with a high signal-to-noise ratio (>1), low relative local background, and MFI > [MFI of local background spots + 2 standard deviations] were marked as positive spots. Background correction was performed on a per-block (7 × 7 block of nanowell) basis using a custom-written script in MATLAB (R2010b; MathWorks, Natick, MA).

### D.1.4 Calculation of the classification accuracy of cellular barcoding

Groups of barcoded cells were loaded into separate wells of a 96-well flat-bottom plate; each well contained a collection of cells with a single, known barcode. Calcein violet (2 µM) was then added to identify viable cells. The cells in each well were imaged, and the intensities of each viable cell’s barcoding dyes were determined. Images were acquired within 30 min of when the images were acquired in the microengraving assays measuring secretion. The data from all cells were then pooled to produce histograms of the distribution of fluorescence intensities for each barcoding dye. Barcode classifications for each cell were assigned based on the intensity thresholds determined from these histograms.

The classification accuracy was calculated as the percentage of cells that received a given barcode (i.e., total number of viable cells in a given well) that were correctly classified as having the given barcode. For example, consider a case in which barcode 1 was applied to a group of cells, which were then loaded into a single well of a 96-well plate. If 100 total cells were analyzed from this well, and if the imaging and analysis procedure classified 98 of these cells as being labeled with barcode 1, then a classification accuracy of 98% would be assigned to barcode 1.

The analysis described above was performed by imaging cells with uniformly applied barcodes in separate wells of a 96-well plate. In actual experiments, however, cells with different barcodes are mixed and imaged in nanowells. In these mixed settings, it is possible that two adjacent cells with different barcodes could be mis-
classified as one double-positive cell. Therefore, to quantify the accuracy of classifying double-positive cells in typical nanowell experiments, we manually reviewed the images of randomly selected putative double-positive cells and recorded how frequently their classification as a double-positive cell was incorrect.
Figure D-1: **Automated counting of viable cells.** Representative image of automated segmentation and counting of cells using Enumerator (written in MATLAB). The positions of the wells are determined from the transmitted light image (not shown), and the segmentation of the cells within the wells is determined from the fluorescence signal of the viability dye (calcein violet; shown in white). Red boxes mark cells that were identified by the segmentation algorithm. The barcode of each identified cell is determined from the intensities of the fluorescent cellular barcoding dyes associated with the cell (not shown).
Table D.1: Classification accuracy for double-positive cells

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Figure D-2: **Intensities of secretion in dye-rotation experiments.** Intensities of secretion from secretion-positive single cells that were exposed to a uniform stimulation (PMA/ionomycin) and labeled with (A) antibody-based barcodes, (B) cytosolic barcodes, or (C) streptavidin-based barcodes. Boxes indicate the median and the 25th and 75th percentiles, and whiskers indicate the min and max. MFI, median fluorescence intensity. *$P < 0.05$, Kruskal-Wallis test followed by Dunn’s post-test. Note: The borderline-significant ($P = 0.039$) difference in the intensity of secreted IL-2 from cells labeled with the streptavidin-based barcodes was only observed in one of nine replicates of the microengraving process; in all other replicates, there was no significant difference ($P > 0.05$) among the streptavidin-based barcodes.
Bibliography


