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Polyfunctional responses by human T cells result from sequential release of cytokines

Qing Han¹, Neda Bagheri¹, Elizabeth M. Bradshaw¹, David A. Hafler¹,d,e, Douglas A. Lauffenburger¹,²,e, and J. Christopher Lovee,g

¹Department of Chemical Engineering, Koch Institute for Integrative Cancer Research, and ²Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139; ³Brigham and Women’s Hospital, Boston, MA 02115; ⁴Department of Neurology and Immunobiology, Yale University, New Haven, CT 06520; and ⁵The Eli and Edythe L. Broad Institute, Cambridge, MA 02142

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The release of cytokines by T cells defines a significant part of their functional activity in vivo, and their ability to produce multiple cytokines has been associated with beneficial immune responses. To date, time-integrated end-point measurements have obscured whether these polyfunctional states arise from the simultaneous or sequential release of cytokines. Here, we used serial, time-dependent, single-cell analysis of primary human T cells to resolve the temporal dynamics of cytokine secretion from individual cells after activation ex vivo. We show that multifunctional, Th1-skewed cytokine responses (IFN-γ, IL-2, TNFα) are initiated asynchronously, but the ensuing dynamic trajectories of these responses evolve programmatically in a sequential manner. That is, cells predominantly release one of these cytokines at a time rather than maintain active secretion of multiple cytokines simultaneously. Furthermore, these dynamic trajectories are strongly associated with the various states of cell differentiation suggesting that transient programmatic activities of many individual T cells contribute to sustained, population-level responses. The trajectories of responses by single cells may also provide unique, time-dependent signatures for immune monitoring that are less compromised by the timing and duration of integrated measures.

microengraving | multifunctionality | dynamical systems | computational biology

T cells play a significant role in adaptive immune responses to infectious diseases and in the pathogenesis of inflammatory diseases (1). Determining their characteristic diversity remains a central goal for defining immunological signatures that indicate the status of human diseases or responses to interventions like vaccines (2). T cells are typically classified by their state of differentiation based on surface-expressed glycoproteins (e.g., CD3, CD8, CD45RA, CCR7) (3) and then assigned a functional state (e.g., Th1, Th2, Th17) based on their ability to produce one or more cytokines within specific groups (4). Efforts to improve immune monitoring have focused on understanding the phenotypes and functions that reflect effective T-cell responses to diseases and clinical interventions, but these correlations have remained imperfect thus far.

Both the magnitude and quality of a T-cell response are considered important metrics in evaluating the efficacy of an immune response (2). The number of responsive cells provides a measure of the magnitude, whereas the nature and diversity of the functional responses has been associated with measures of quality. These functions include releasing one or more cytokines that induce proliferation, modulate inflammation, mediate cytolysis of other cells, and inhibit viral replication (1). The production of multiple cytokines by T cells has been associated with productive immune responses to infectious diseases (5–7) and to vaccines (8–10).

The manner in which polyfunctional responses by individual cells contribute to the evolution of an immune response at a population level is not well understood. The types and concentrations of cytokines in the extracellular milieu, and percentages of cells producing them, are known to shift globally over time (11, 12). Indeed, the production of both IL-2 and IFN-γ by CD4+ T cells in vivo has been shown to begin within hours of stimulation and wane after 16–18 h (13, 14). It has not been possible, however, to determine whether cells release multiple cytokines simultaneously, or sequentially, in time because techniques such as intracellular cytokine staining (ICS) and multiparametric ELISPOT provide only integrative, endpoint measures (15–18). Therefore, resolving when activated T cells initiate the release of cytokines, and how their responses evolve in time, should provide fundamental insight into how individual cells dynamically modulate intercellular signals to affect population-level responses toward pathological conditions or clinical interventions.

Here we examine how the synchrony and evolution of secreted cytokines varies upon activation among different subsets of primary human CD3⁺ T cells isolated from peripheral blood. Using a combination of imaging cytometry and quantitative single-cell analysis of secreted cytokines, we monitored the release of three Th1-skewed cytokines (IFN-γ, IL-2, and TNFα) over time. We find that T cells initiate the release of cytokines at different points in time upon stimulation. Furthermore, most of these cells initiate secretion in a monofunctional state. Computational analyses of these data indicate that the simultaneous release of the measured cytokines is short-lived and that cells follow programmatic, rather than random, patterns of release. Moreover, T-cell receptor (TCR)-dependent activation does not change the nature of these trajectories. Finally, we present evidence that these trajectories, rather than initial time of secretion or the overall integrated response, associate closely with the differentiated state of the cell. Together, observations of distributed activation and evolving release suggest how single T cells may use time-dependent mechanisms to evolve population-level responses and how dynamic monitoring of immune cells may improve profiling functional responses associated with immune status relative to integrated, endpoint measurements (19).

Results

Serial Microengraving Quantifies Dynamic Rates of Cytokine Secretion from Single T Cells. We used a dense array of subnanoliter wells (nanowells) to isolate CD3⁺ T cells from the peripheral blood of healthy subjects. After distributing cells into the array at a density of approximately one cell per well, we imaged each well by automated fluorescence microscopy to determine the occupancy, viability, and

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differentiated state of each T cell (classified by CD8, CD45RA, and CCR7) (Fig. 1A and Fig. S1). Each array comprised 84,672 wells and yielded over 10,000 individual cells per experiment.

The cells in the array were then activated by a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin (20). We chose this TCR-independent stimulation initially to probe a broad range of responses. We used serial microengraving—a non-destructive, quantitative method for sampling the supernatants from each cell in the array (SI Text) (21)—to measure the paracrine rates of secretion for three cytokines associated with Th1-skewed responses (IFN-γ, IL-2, and TNFα) every 2 h during sustained stimulation (Fig. 1A). This process generates 30 data points per cell over 16 h that describe the temporal release of cytokines from eight distinct CD3+ T-cell subsets (Fig. 1B and Figs. S1 and S2).

The data collected from activated, viable single cells revealed that the secretion of cytokines occurred in a dynamic and heterogeneous manner (Fig. 1C). Aggregation of the responses showed the numbers of cells secreting IL-2 and IFN-γ increased over time, whereas those secreting TNFα diminished (Fig. 2A and Fig. S3A–D). The secretion of IL-2 was also more prevalent among CD8− T cells than CD8+ T cells. Together, these basic trends were consistent with those determined by bulk analysis of the order and timing of cytokine responses using ICS and ELISA (12, 22). The mean rates of secretion measured for each cytokine by microengraving were also consistent with those estimated from a combination of ICS and Luminex (Fig. S4A).

**Initial Release of Cytokines Varies Temporally Among Activated T Cells.** One interesting observation was that new cohorts of T cells sharply transitioned into active secretion during each sampling period. These asynchronous, apparently stochastic, events were biphasic, concentrated at ≈2–6 h and 12–16 h (Fig. 2B). Interestingly, there was no statistical association between the timing of initiation and specific subsets, although memory T cells (CD45RA−) tended to respond most often during the first burst of activity (Fig. S3E and Table S1).

To confirm that these variations were not influenced by isolated activation, we also measured secretion from cells stimulated in a bulk culture. The frequencies of secreting cells were consistent when measured by microengraving 6–7 h after stimulation either in bulk (10.7%) or on-chip (13.6%). These frequencies were also smaller than the accumulated numbers of cells measured by ICS over 7 h, further supporting temporal variance in activation (Fig. S4B). The diversity of differentiated subsets present ex vivo could also impact the observed timing of initial release so we also measured the secretion from an in vitro-expanded human T-cell clone (Fig. S5). These cells exhibited similar asynchronous release of cytokines under persistent stimulation. Variability in the expression levels of kinases, transcription factors, and other signaling proteins (ERK, NFAT, SHP-1) (23, 24), along with slow epigenetic events such as chromatin remodeling near transcription factor binding sites that promote production of cytokines (25, 26), may contribute to these observed temporal distributions of initial release.

**Simultaneous Release of Multiple Cytokines Is Transient.** Although ICS enumerates cells that produce multiple cytokines over time, it cannot reveal the lifetime, persistence, or concomitance of these productions. Our experiments here demonstrated that most cells (≈90%) first initiate secretion in a monofunctional manner, releasing only a single cytokine. Further, the frequency

![Diagram showing cytokine secretion dynamics for individual T cells upon activation.](image)

**Fig. 1.** Cytokine secretion dynamics for individual T cells upon activation. (A) Illustration of serial microengraving to monitor cytokine secretion by viable single T cells in time. (B) Representative micrographs of data evaluating viability (Calcein and SYTOX); phenotype (CD8, CD45RA, and CCR7); and TNFα (blue), IL-2 (red), and IFN-γ (green) secretion. Blue squares outline positive events. (C) Cytokine secretion kinetics of 3,015 viable T cells. Each row within each block reflects the dynamic activity of an individual T cell over time. The color wheel illustrates the type and relative magnitude of secreted cytokines; inactivity is black. Block columns and block rows organize cytokine profiles by initial time of activity and differentiated cell types, respectively. Kinetic profiles are ordered within each block according to cytokine function. These data are representative from three independent experiments.
of multifunctional states during any single sampling period was significantly lower than that seen by integrating these data across time (Fig. 2A and Fig. S3F). These observations suggest T cells are more likely to secrete multiple cytokines sequentially than simultaneously.

We found that cells secreting multiple cytokines simultaneously were more likely to change their functional states than those releasing individual cytokines (Fig. 2C). Only cells that secreted IFN-γ or IL-2 showed significant persistence of their functional states. The average lifetimes of states in which two or more cytokines were secreted simultaneously were 1.5- to 2-fold shorter than those of IFN-γ or IL-2 alone (Fig. 2D). Cells that initiated secretion within 4 h of stimulation were more likely to produce multiple cytokines in total, either simultaneously or sequentially (Fig. S3G). Together, these results demonstrate that the simultaneous secretion of two or more Th1-associated cytokines likely occurs as a transition between states, and that the secretory responses by T cells evolve dynamically during sustained, TCR-independent activation.

**Multifunctional Th1 Responses Evolve with Time.** To identify the most common transitions among functional states, we quantified the likelihood that a cell in a secretory state at time tN would transition to another state 2 h later, tN+2h (Fig. 3A). The most probable outcomes observed here were either retaining the current state or downgrading the number of cytokines secreted. For example, the release of TNFα in combination with either IFN-γ or IL-2 commonly resolved to the secretion of IFN-γ or IL-2 alone. These analyses further confirm that cytokine secretion by individual cells occurs in a predominantly sequential manner, with multifunctional release arising as a transient state.

We then computed the corresponding Z scores for these state transitions relative to randomly permuted datasets to evaluate whether certain transitions occurred more or less commonly than expected by chance (Fig. 3B). As anticipated, persistence of individual secretory states was significant, confirming that cells actively sustain specific functional states. Other transitions were significantly underrepresented. For instance, observed transitions between IFN-γ and IL-2 occurred less frequently than expected by chance. This result is consistent with observations that IFN-γ expression, controlled by the transcription factor T-bet, suppresses the bulk production of IL-2 by lymphoma cells activated by PMA/ionomycin (27). We anticipate that identifying dominant individual-cell secretory transitions may offer new insights on the regulation of cytokine signaling and provide a means to predict T-cell responses.

**T Cells Exhibit Programmatic Trajectories of Cytokine Secretion.** The global transition matrices suggested that the trajectories of secretory states among cells evolve with identifiable, deterministic programs, rather than stochastic or idiosyncratic courses. That is, the set of trajectories observed is small relative to the number of possible trajectories for the three cytokines (>10^6). To test this hypothesis, we investigated the cytokine trajectories that emerged from clustering the first three time-aligned data points by self-organizing maps (SOMs) (Figs. S6 and S7). For each CD8+ T-cell subset, the optimal number of clusters was determined by evaluating the explained variance (elbow criterion) (28) (Fig. S7A). Metaclusters were determined by further SOMs and qualitative alignment of similar clusters (Fig. 3C and Fig. S7B and C).

The dominant trajectories exhibited either persistent secretion of individual cytokines (e.g., IL-2, IFN-γ) or a transition from a single functional state to another (e.g., TNFα → IL-2; TNFα → IFN-γ). Memory T cells (CD45RA−) used the most diverse sets of states, with a small, but significant, bias toward TNFα secreting states among the effector memory (CCR7−) cells, whereas CD45RA+ cells predominantly exhibited a short burst of IFN-γ. These results support models for T-cell differentiation where T cells maintain transient memory for gene expression resulting from chromatin remodeling (26), and also suggest that some subsets of T cells from all differentiated populations can release limited bursts of IFN-γ within 2 h of initial activation.

**Dynamic Cytokine Trajectories Can Discriminate T-Cell Subsets.** We next considered whether the observed sequential cytokine trajectories could distinguish different subsets of cells (effector memory, central memory, effector, and naïve) more effectively than time-integrated data, which may fail to resolve differences in how multifunctional responses are reached. Using principal component analysis (PCA), in combination with feature selection, we identified unique subspaces that best segregated subsets in specified training data. These subspaces were subsequently used to classify cells based on their dynamic cytokine profiles. Using raw CD8+ T-cell data, we could discriminate among the four subsets more accurately (41 ± 1%, percent correct classification) than random assignment (25%) (Fig. 3D). Integrating the data over 6 h (i.e., approximating ICS) reduced the accuracy of classification to 33 ± 1%. Remarkably, aligning the trajectories...
of individual cells according to the initial time of cytokine release dramatically improved the accuracy of classification to 58 ± 4%. We also found that classification using time-aligned data improved monotonically with the temporal length of the trajectories, especially for naive and effector cells (Fig. 3E and Fig. S8A). Sensitivity analysis for the binary classification of subsets (based on receiver operating characteristic curves) confirmed that effector memory and central memory cells were challenging to discriminate based on their functional profiles (Fig. S8B), suggesting that there are limited differences between the ranges of dynamic cytokine responses for these two subsets, and that local microenvironments along with receptor-mediated signaling likely modulate context-specific responses. Further resolution of the phenotypic diversity within memory cells may also improve their classification (3).

**TCR-Dependent Activation Induces Similar Programmed Responses.**

Whereas the stimulation of T cells in a TCR-independent manner provided a view of the accessible trajectories of secretory states, activation of T cells in vivo occurs through the engagement of TCRs with cognate antigens presented in class I or II major histocompatibility complexes (MHC) and costimulatory molecules such as CD28 (29). To determine whether the dynamics of cytokine secretion after PMA/ionomycin stimulation were consistent with TCR-dependent stimulation, we compared the responses of primary T cells subjected to each condition during the period in which all functional states and transitions were most prevalent (2–10 h). We coincubated CD3+ T cells with beads bearing anti-CD3 and anti-CD28 as a homogeneous surrogate for antigen-presenting cells (APCs) (4 ± 2 beads per well), and monitored the dynamic evolution of their secretory states; T cells predominantly contacted beads in the wells within 1 h (Movie S1). Qualitatively, the responses measured were similar to those observed during TCR-independent stimulation (Fig. 4A). Different populations of cells again initiated secretion of cytokines throughout the period of observation, and most cells did not begin in a multifunctional state.

To compare the differences in responses between stimuli directly, we monitored the cytokine responses from T cells isolated from the same subjects. TCR-dependent stimulation produced more cells secreting IL-2, and fewer secreting TNFα, than those stimulated with PMA/ionomycin (Fig. 4B). TCR-dependent activation also favored fast, limited bursts of secretion rather than sustained release, despite persistent stimulation (Fig. 4C). This response is qualitatively consistent with the finite temporal persistence of phosphorylated ERK observed by flow cytometry in mouse T cells after activation (30). Regulation of persistent TCR-dependent signals to allow only transient release of cytokines suggests another mechanism for limiting the effects of indiscriminate activation and supports in vivo observations that multiple serial encounters are often required to induce activation (31).

For both stimulations, cells secreting a single cytokine were more likely to preserve that functional response than cells releasing multiple cytokines (Fig. 4D). The number of cells preserving functions was generally higher after TCR-dependent stimulation and may be a consequence of the limited bursts of activity (Fig. 4E). Surprisingly, both stimuli induced similar trajectories, differing only in the frequencies of observed states (Fig. 4F). Although stimulation via the TCR appears to alter the timing and persistence of specific secretory states, the programmatic
trajectories of secretory states after activation do not appear to depend strongly on TCR-mediated signaling.

Discussion
We have presented an intensive experimental characterization of the dynamic evolution of cytokine secretion exhibited by individual human T cells. Together, these data reveal that cytokine responses evolve along a limited set of largely deterministic courses regardless of the initiating cue, and the accessibility of the particular courses vary among differentiated subsets of T cells. These dynamic responses more accurately distinguish the subsets than either the timing of activation or the cumulative cytokine response. The temporal diversity in functional phenotypes observed here have implications for both monitoring immune responses and the behaviors of the cellular networks comprising the immune system.

The asynchronous activation and programmatic evolution of responses by single T cells have practical consequences for immune monitoring. These time-dependent attributes represent sources of intrinsic noise for common integrative measurements like ELISpot or ICS. Such assays are likely influenced by both the timing and duration of sampling, and the degree of functionality assigned to a cell may be misclassified within a specific window of time. Because distinct classes of trajectories for secretion were consistently identified across samples from multiple donors, the frequencies of these trajectory classes in patient samples may provide time-dependent cellular signatures associated with particular clinical conditions.

More broadly, our findings suggest another means for enabling robustness within the network of cells comprising the immune system. The maintenance of homeostatic stability, while concurrently monitoring environmental perturbations, is an essential property of the immune system—one that is believed to derive from modularity of components and incorporation of control via feedback (32, 33). The capability of T cells to access multiple programs for cytokine release allows diverse and dynamically evolvable contributions to the sustained accumulation of cytokines in the global milieu, and such behavior represents an intriguing manifestation of modularity, cell-to-cell communication, and feedback. The adaptability of multicellular systems to temporal and environmental fluctuations is yet another quality that improves when the collective population comprises phenotypically diverse individuals (34–36). Dynamically evolving functional responses by T cells, therefore, suggest an alternate form of functional plasticity (37) and may provide yet another means for the immune system to adaptively respond to perturbations.

Temporal phenotypic diversity among T cells resonates with other recent experimental and computational observations. For example, the activation of individual T cells does not occur homogeneously upon stimulation—even isogenic ones—but rather activate in a digital (on/off) manner, resulting from variations in transcriptional activity or gene accessibility (25, 26), the numbers and dynamic expression of signaling receptors (e.g., CD8, IL-2R), and regulators of both positive and negative feedback loops (e.g., ERK, SHP-1, and RAS) (23, 30, 38, 39). Expression of IL-2 and IFN-γ in human CD4+ T cells has also been shown to depend on the categorical translocation of the transcription factor NFATc2 (24). Our results revealing time-dependent variations in secretion do not directly resolve the relationship between this functional outcome and variances within the molecular signaling networks that regulate the production and subsequent release of cytokines; further characterization of this linkage could inform the regulatory mechanisms involved in the distinct trajectories measured.

A report by Feinerman et al. (39) supports the notion that the transient nature of cytokine release discerned here can offer opportunities for feedback and modulation of inputs from proximal cells. Incorporating APCs in a single-cell coculture system would further refine our dynamic single-cell measures to reveal the effects of both antigen-specificity and cell–cell interactions on the evolution of paracrine responses. We anticipate that dynamic single-cell analysis of cellular functional responses should help...
evaluate the nature and evolution of intercellular interactions present in biological systems such as lymphatic tissues, tumor microenvironments, and stromal niches for stem cells.

Materials and Methods

Dynamic Single-Cell Analysis of Cytokine Release. Arrays of 1-mm-thick polydimethylsiloxane (PDMS) nanowells (50 μm or 30 μm) were manufactured by using a custom-built mold and adhered directly to a 3-inch × 1-inch glass slide. Primary human CD3+ T cells were isolated from peripheral blood of healthy donors, stained for CD8, CD45RA, CC57, and viability (Calcein violet), then loaded into nanowells at a density of 1×10^6 cells per well. The array of nanowells was imaged by automated epifluorescence microscopy (Zeiss). After imaging, the array was cultured in serum-free HL-1 complete media supplemented with 10 ng/mL PMA and 1 μg/mL ionomycin, at 37 °C with 5% CO2. Cytokine secretion was measured 2 h after initial stimulation and repeated every 2 h (21). During each cycle (2 h), IFN-γ, IL-2, and TNFα from each well was captured by microengraving with a glass slide supporting corresponding antibodies for 1 h, followed by culturing the nanowells in media for another hour. After the kinetic measurements, cells were stained in situ with a viability marker (Calcein) and a dead cell marker (SYTOX green) and reimaged. Glass slides with captured cytokines were probed with fluorescence-labeled detection antibodies and imaged. TCR-mediated responses were measured by coculturing T cells with anti-CD3/CD28 Dynabeads.

Data Analysis. Secretion data and cell phenotypes were extracted from collected images and then combined according to the unique ID for each nanowell. Wells occupied with single live cells before and after serial microengraving that yielded cytokine secretion at any of the measured time points were selected for analysis.

Computational and Statistical Analysis. SOMs were used to cluster similar dynamic profiles from individual T-cell responses. Principal component analysis (PCA) was used to classify T-cell subsets based on their functional profiles. Receiver operating characteristic curves were computed to evaluate the sensitivity of classification.

Additional Methods. Additional descriptions for all methods are available in SI Materials and Methods, including fabrication of nanowells, functionalization of glass, postprocessing of printed arrays, data processing, and computational analysis.

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