An animated landscape representation of CD4$^+$ T-cell differentiation, variability, and plasticity: Insights into the behavior of populations versus cells

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Recent advances in understanding CD4$^+$ T-cell differentiation suggest that previous models of a few distinct, stable effector phenotypes were too simplistic. Although several well-characterized phenotypes are still recognized, some states display plasticity, and intermediate phenotypes exist. As a framework for reexamining these concepts, we use Waddington's landscape paradigm, augmented with explicit consideration of stochastic variations. Our animation program "LAVA" visualizes T-cell differentiation as cells moving across a landscape of hills and valleys, leading to attractor basins representing stable or semistable differentiation states. The model illustrates several principles, including: (i) cell populations may behave more predictably than individual cells; (ii) analogous to reticulate evolution, differentiation may proceed through a network of interconnected states, rather than a single well-defined pathway; (iii) relatively minor changes in the barriers between attractor basins can change the stability or plasticity of a population; (iv) intrapopulation variability of gene expression may be an important regulator of differentiation, rather than inconsequential noise; (v) the behavior of some populations may be defined mainly by the behavior of outlier cells. While not a quantitative representation of actual differentiation, our model is intended to provoke discussion of T-cell differentiation pathways, particularly highlighting a probabilistic view of transitions between states.

Keywords: CD4$^+$ T cells · Cell differentiation · Cytokines · Modeling

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Introduction

Purpose of review

CD4$^+$ T-cell differentiation into effector subtypes has been extensively studied, and much is known about the molecular pathways influencing differentiation into subsets, such as Th1, Th2, Th17, and others. However, there has always been a slight uneasiness about the heterogeneity of the cell populations at different stages of this process—differentiation is often a trend rather than a uniform alteration of all cells in the population. We will briefly discuss CD4$^+$ T-cell differentiation from naïve into effector cells, with reference to many excellent recent reviews in this area, then suggest the use of Waddington's "epigenetic landscape" as a metaphor for visualizing and better comprehending the behavior

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of individual cells and how these contribute to the behavior of populations.

Landscape models

In order to integrate the stochastic and regulated patterns of gene expression within the same conceptual framework, and explain their respective manifestation in flow cytometry as either broad continuous distributions or discrete clusters, it is useful to think of cell state changes as a process on a landscape. Waddington (1957) introduced the idea that the differentiation of cells could be represented as the movement of cells across a landscape, representing a continuum of states [1] in which the cell states change according to the shape of the terrain and the force of gravity: rolling down valleys and accumulating at the lowest points accessible from a given position. While originally introduced as an intuitive metaphor, Waddington's landscape is more than just a useful visualization: it provides access to abstract mathematical principles that describe how gene regulatory networks (GRNs) govern the changes of gene expression patterns of each cell as they differentiate [2]. As long as one obeys common principles shared with the elementary properties of landscape topographies, the properties of this visualization are readily adapted to known qualitative properties of biological systems even without detailed knowledge of the regulatory pathways that produce the landscape.

Although quantitative, accurate landscape models can in principle be constructed if one knows the structure of the relevant GRN and the numerical parameters that specify the interactions in the GRN [3], these would be very high-dimensional because of the large number of interacting genes (and their products) that influence T-cell differentiation. We will not attempt to describe such a quantitative landscape model in this review—rather, to maintain the simplicity of the landscape visualization, we use a 3D landscape metaphor to bring forward conceptual issues for discussion [4]. Our goal is to provoke discussion regarding the behavior of populations versus cells, and stimulate experimentation to more conclusively resolve these issues.

Types of effector T-cell diversity, and regulatory mechanisms controlling subtype differentiation

Before introducing our landscape model, we briefly summarize current knowledge of T-cell differentiation states and pathways. This area has been covered by many excellent recent reviews [5–10], therefore we summarize the information necessary to frame the questions that we address in the landscape model.

The main themes — recognized "stable" phenotypes

How many discrete subsets of effector CD4$^+$ T cells exist? Since the initial recognition of the Th1/Th2 dichotomy [11–13], several other T-cell subtypes have been identified based on cytokine

secretion and effector functions, including regulatory T (Treg) cells [14–16], Th17 cells [17, 18], primed precursor T (Thpp) cells [19–21], Th9 cells [22, 23], Th22 cells [24, 25], and also follicular helper T (Tfh) cells [26–28], although it is not yet clear whether Tfh cells are truly a distinct lineage or derive from cells already committed to Th1, Th2, or Th17 lineages [6, 29–31].

Differentiated CD4$^+$ T cells can also be classified, based on activation markers, tissue-homing specificity, and proliferative potential into short-lived effector cells and memory precursor effector cells, the latter further differentiating into central memory (Tcm), effector memory (Tem), and tissue-resident memory (Trm) cells [10, 32–38]. While these memory/effector categories may not be appropriate for classifying Treg, Tfh, and memory stem cells [39], the effector and memory subsets may all include committed Th1, Th2, and Th17 cells, as well as Thpp, Th9, and Th22 cells.

Regulation of differentiation of major subsets

Cytokines from dendritic cells, other innate immune cells, and CD4$^+$ T cells themselves are the dominant regulators of the differentiation of naïve CD4$^+$ T cells into Th1, Th2, Th17, and other subsets [40, 41]. IFN-γ, IFN-α, and IL-12 induce Th1 differentiation, IL-4 is the primary inducer of differentiation into Th2 cells, and combinations of IL-1, TGF-β, and IL-6 induce Th17 differentiation. Overlaid on these cytokine effects, strength of TCR signaling, and possibly the kinetics of antigen exposure, can also influence polarization, with strong signals generating Th1, Th17, and Tfh responses, and weaker stimulation generating Th2 responses [42–47]. Other soluble factors, including hormones (progesterone, estradiol), eicosanoids, retinoids, and nucleosides, also play a role. The major differentiation signals are described in Fig. 1 of [7].

Differentiation into effector T cells is a complex process that takes a few days for complete commitment, and may pass through several intermediate stages [6]. The committed phenotypes are stabilized by a network of mutually inhibitory transcription factors, and epigenetic modifications of the relevant cytokine genes, as well as the transcription factors that dominate the regulation of each T-cell type [5, 48, 49]. In addition to the regulation of the differentiation pathway, it is important to consider the regulation of the stability of the resulting phenotype. This may be a complex network of positive and negative regulatory loops, operating through transcription factor networks, epigenetic (DNA and chromatin) modifications, and miRNA regulatory networks. The net result is the existence of several moderately stable states.

Variations on the basic themes

More recently, evidence has accumulated that there is more flexibility/plasticity of the main effector phenotypes than previously surmised, particularly in vivo, and particularly in humans (reviewed in [50–53]). Treg and Th17 cells in particular show considerable potential for further differentiation, for example,
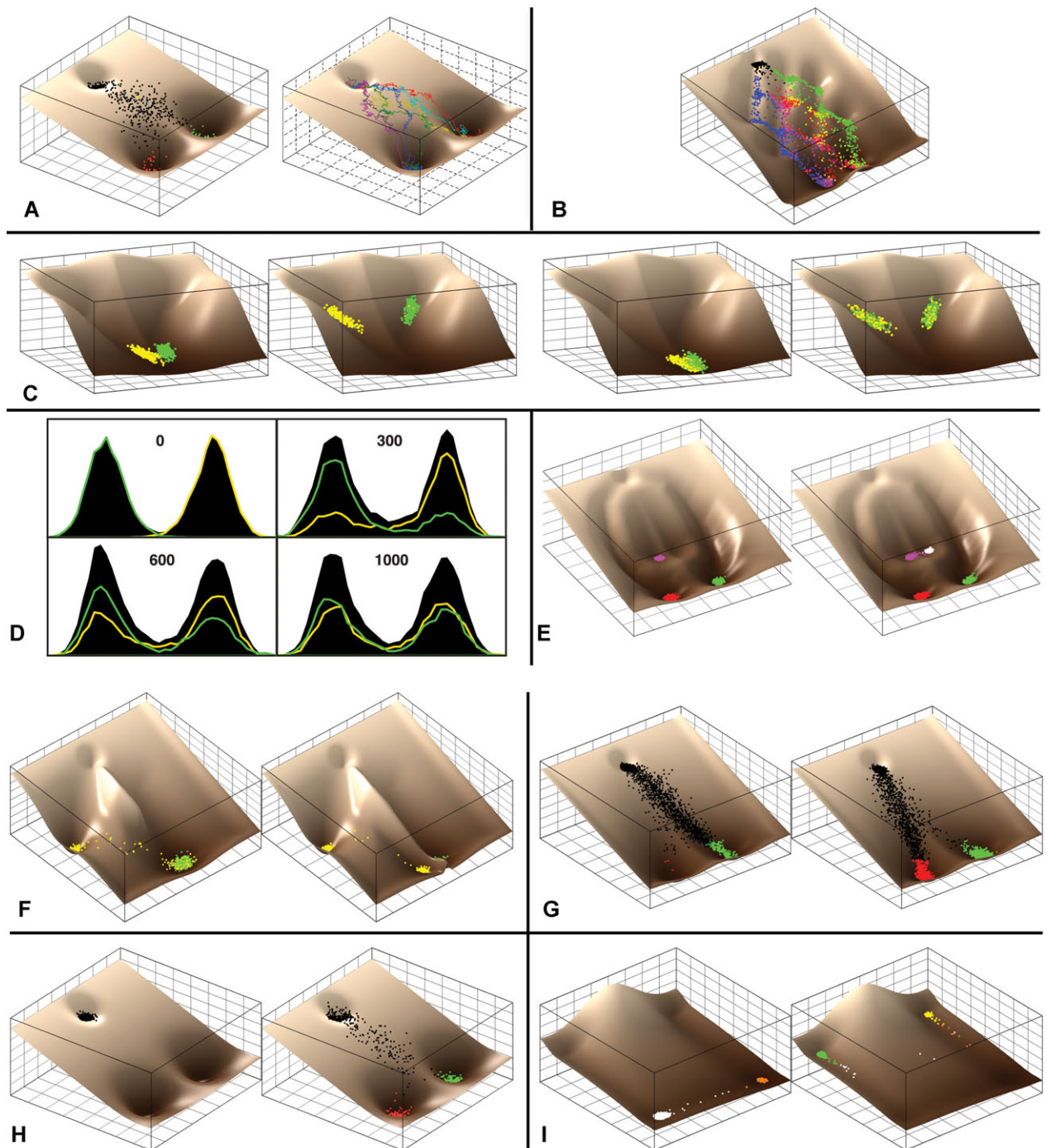
Figure 1. Static images from LAVA animations. These images are taken from Supporting Information Animations 1–9. (A) A frame from the middle of Animation 1 shows differentiation of naïve CD4$^+$ T cells into two well-defined phenotypes, Th1 and Th2 (left). Seven individual tracks illustrating the variable paths taken by different cells are shown (right). (B) Animation 2 suggests that reticulate differentiation can occur, with both Th1 and Th2 basins acquiring cells that have taken different paths, indicated by different colors, through several intermediate metastable states. C) Animation 3 shows two cycles of activation of Th1 cells to secrete cytokines. The images show the cytokine pattern histories of cells with a brief (left pair) or extended (right pair) period of equilibration between stimulations. (D) Animation 4 shows the equilibration of two starting populations within two overlapping basins. The histograms show the two initial populations (green and yellow) and the total population (black) after 0, 300, 600, and 1000 cycles of the simulation. (E) Animation 5 shows the plasticity of two overlapping attractors (Th17 and Treg) when a differentiation-inducing "wind" is applied. (F) Animation 6 illustrates parallel versus convergent differentiation of Th17 and Th1 phenotypes. The images show the final cell positions for the convergent (left) and parallel (right) cases. (G) Animation 7 shows the effect of positive feedback on Th2 differentiation. The images show early Th1-biased (left) and late Th2-biased (right) stages, as driven by the changing "wind." (H) Animation 8 shows that a low level of stochastic variability retains all cells in the initial naïve state (left), whereas higher variability alone can induce cells to escape down the differentiation path (right). (I) Animation 9 illustrates very slow leakage of naïve T cells toward an inflammation-induced phenotype ("aged naïve" T cells, left), and the subsequent stimulation of the two populations into separate activation states by an exogenous "wind" (right).

Treg → Th17 and Th17 → Th1-like transitions [54–61]. Th1 and Th2 cells are also more flexible than originally thought. Th1 cells can convert to IL-4-producing cells during a strongly Th2-polarizing helminth infection [62], Th2 cells can secrete IL-17 in asthma models [63], lymphocytic choriomeningitis virus (LCMV) infection reprograms Th2 cells to GATA3$^+$Tbet$^+$ "Th2+1" long-lived cells that secrete IFN-γ and IL-4 [64], and parasite infection can prime stable GATA3$^+$Tbet$^+$ Th1/Th2 hybrid cells [65].

In these transitions, do the cells convert fully to a different state, for example, Th1 → Th2, or do the cells acquire some characteristics of the other state, but retain part of their original identity? Th17 cells that convert to IFN-γ-producing cells [66] nevertheless retain chromosomal marks and cytokines characteristic of the original Th17 cells. This separate identity of "Th1-like Th17 cells" is consistent with the possibility that Th17 effectors may be derived from a distinct lineage of naïve cells expressing CD161 [67, 68]. In some cases, it remains to be determined whether full conversion is achieved, for example, in the Th1 conversion to IL-4-producing cells in helminth infection [62].

In addition to the familiar linear branched differentiation diagrams, it may also be useful to describe four major subsets of CD4$^+$ T cells—Th1, Th2, Th17, and Treg cells—each of which can take on characteristics of the other subsets, or further characteristics. Cells expressing the Th1, Th2, or Th17 cytokines and transcription factors can all acquire characteristics of Tfh (follicular homing, *bcl6* expression, B-cell help), as illustrated, for example, in figure 3 of [29]. Associated with the modified phenotypes, epigenetic marks on cytokine genes can be altered. This may be facilitated by the ambivalent status of epigenetic regulation of the characteristic transcription factors—*Tbx21, Gata3, Bcl6* can have both permissive and nonpermissive marks even in differentiated cells [5, 69].

Short-term environmentally induced changes

In general, the processes described above represent nonreversible differentiation. T effector cells can also undergo short-term, reversible changes in their expression patterns of cytokines and other effector molecules. Although in some cases it is not yet known whether some changes are reversible, for this review we distinguish between stable differentiation versus reversible modulation to a state that reverts to the original state after withdrawal of the inducing agent. Environmental factors present during activation can alter cytokine patterns, for example, cAMP-elevating agonists, such as prostaglandin E2 and adenosine, can reduce the expression of most cytokines, but enhance the synthesis of amphiregulin [70]. IL-12 enhances cytokine synthesis by Th1 cells, and IL-12 and IL-18 induce IFN-γ production even in the absence of TCR signals [71, 72]. Location-specific alterations in T-cell function occur in CD4$^+$ effector cells during localized infection [73], although it is not yet clear whether these represent reversible modulation, selective recruitment, or further differentiation. CD8$^+$ resident memory (Trm) cells probably develop locally from circulating precursors [74].

Variation within a recognized phenotype

Single-cell data from flow cytometry and PCR analysis suggest considerable heterogeneity of gene expression, or at least gene product levels. Even when apparently homogeneous populations are isolated by high-resolution automated clustering algorithms [75], measured markers for most populations spread across one or more decades of expression in a distribution that appears approximately symmetrical and Gaussian on a log scale. Importantly, most markers vary independently within a population, unless these markers are part of the same multicomponent complex, for example, CD8α and CD8β, or share a regulatory pathway. To put this level of variation into perspective, for some genes there is a recognizable haploinsufficiency phenotype in +/− mice compared to +/+ mice. If twofold changes in expression levels at the *population* level can have consequences, what is the significance of the normal ten-fold range among individual cells? Does this variation just represent noisy fluctuations? As suggested by Hodgkin and colleagues, variation within a population may contribute to cell behavior by providing a more graded, regulatable response [76].

A more dichotomous example of this type of diversity is the well-known variable expression of several cytokine genes, both at the level of on/off expression, for example, Th1 cells may be IL-2$^+$ IFN-γ^+, IL-2$^+$ IFN-γ^-, IL-2$^-$ IFN-γ^+, or IL-2$^-$ IFN-γ^- during any single stimulation cycle [77], and also in mono- and diallelic expression patterns [78–82]. These two phenomena may be linked, as stochastic expression of individual alleles would predict a mixture of positive and negative cells. Mechanisms underlying stochastic expression are currently unknown, but could involve threshold effects [83], competitive binding of positive and negative transcription factors, or stochastic epigenetic modifications [84, 85].

Summary of T-cell diversity

The current picture of CD4$^+$ T-cell differentiation retains the idea of specialized effector phenotypes with different functions, but also incorporates a high level of diversity of cell populations and transitions. We believe that this diversity may be best understood at the level of populations, rather than individual cells, and that a more probabilistic approach may help to make sense of the rapidly expanding set of phenotypes and transitions. We will explore this using an animated landscape visualization, which may be an ideal metaphor for this discussion.

Our landscape model for T-cell differentiation

The basic idea of the landscape model [2] is that it captures the constraints imposed by the regulatory network in the cell, which dictates how cells can change their properties. The regulatory network can include interactions in transcription, protein phosphorylation, miRNA, epigenetic modifications, glycosylation, protein

and mRNA degradation, and potentially other cell processes. Every position on the landscape represents a potential cell state (or phenotype) as defined by the cell's full regulatory network, aptly pictured by Waddington [1] as a ball poised to roll down the terrain. The animation uses balls to represent cell positions (states) upon the landscape. Thus, any change in cell phenotype, that is, in regulatory state, is embodied by movement of the ball (cell).

Through the implied gravity, the landscape picture captures the intrinsic driving forces for phenotypic change emanating from the regulatory pathways. A ball at each point rolls down the slopes, tending toward a "lower energy" state until it comes (nearly) to rest at the bottom of a basin, where all local deviations are directed uphill against the gravitational potential and therefore disfavored. The basins represent stable "attractor" states that impose no driving force to change the cell phenotype. The region in the landscape that "drains" into a given attractor state constitutes its "basin of attraction" [4, 86]. A central historical thesis in the landscape formalism is that attractor states represent (quasi) discrete, semistable, or stable cell phenotypes. This idea was first proposed by Delbruck [87] and later by Monod and Jacob [88] independent of Waddington's landscape [2]. In the simplest landscape model, the ball rolling down to the attractor states is guided by the topography of the landscape. This trajectory is the developmental path for an individual cell. The landscape, predestined by the regulatory network, constrains how regulatory network profiles can change.

The landscape can also be used to visualize switching between attractors, that is, between distinct phenotypes. In one type of transition, alteration in the parameters that characterize the regulatory network interactions (e.g., by mutation) can change the landscape topography: flattening a hill here, deepening a valley there, etc., thereby influencing the course of the gravity-defined trajectories of the ball/cells.

In this review, we focus on another type of change, triggered by external perturbations, which can be represented by forces that push a cell against the dictate of "gravity" represented by slopes of the landscape. Perturbations can influence the choice of valleys for individual cells traversing the landscape, or even kick cells "uphill," away from the lowest points in attractors, potentially driving them across hills to other attractor states.

The LAVA program for animation of landscape models

To represent the behavior of cells over time as different molecular parameters are varied or as perturbations are imposed, we have developed the MATLAB program LAVA (landscape animation for visualizing attractors) to animate the progression of cells' states across a landscape with time. LAVA uses a set of reasonably simple rules, yet can produce effects that are consistent with biological intuition. The concrete assumptions that underlie our model for T-cell differentiation in principle comply with the fundamental properties of the landscape model, but the specific details are arbitrary. LAVA operates under the following assumptions.

The landscape

In our model parameterization, the landscape represents the characteristics of the interactions of all regulatory networks in the cell, including transcriptional, protein degradation, signaling (phosphorylation, other modifications), DNA modification, miRNA networks, etc. In contrast, external signals (see below) do not affect the shape of the landscape. Every position on the landscape thus represents a unique regulatory network state, and hence a cell phenotypic state. Barring external influences and stochastic variations described below, cells starting in a given state would experience identical gravitational forces and follow the same trajectory. The semistable cell differentiation states within the T-cell lineage, for example, Th1, Th2, are represented by attractor basins. The full landscape of T-cell differentiation that incorporates all regulatory interactions that drive T-cell differentiation into all T-cell subtypes is extremely large, multidimensional, and not fully known, so we show only simplified small components to illustrate our points.

External influences—winds

In addition to the shape of the landscape, movement on the landscape can also be driven by temporary external factors. As the landscape topography remains unchanged, these influences can be represented as "winds" that exert forces on the cells. Winds change the position, that is, state of the cells, hence correspond to the alteration of one or more components of the regulatory networks. Due to their extrinsic origin, winds can defy the slopes of the landscape that reflect internal driving forces. Thus, the net "movement" of cells is due to the integrated effect of the winds and slopes in the landscape. Examples of "winds" could include externally applied cytokines or drugs that transiently alter network components and thereby influence the trajectory of cell phenotype changes, for example, differentiation. Pathogens would also be represented as "winds." Susceptibility to wind can vary with location on the landscape, for example, a cytokine receptor may be expressed only in certain regions of the landscape.

Probabilistic variation

A very important component of our model is the introduction of stochastic fluctuations in gene expression [89]. This is biologically warranted because gene expression is subject to random fluctuations due to the stochastic nature of biochemical reactions in the cell. This produces the variability in gene expression within a population of cells of identical type, as described above. On the landscape such gene expression noise is manifest as small-scale "wiggling" movements of individual cells that are independent of the pull of gravity. However, attractors limit this stochastic variability and cells tend toward the center of the attractor [4, 90]. Collectively, for a cell population the balance between stochastic variability and movement toward an attractor results in a distribution similar to the familiar clusters in flow cytometry. Such

variability has important consequences for the behavior of cell populations. The magnitude of the probabilistic variation can vary in different regions of the landscape and will interact with the "depth" of attractor basins: diffuse populations will result from flatter attractor basins and/or higher stochastic variability in regulatory network components.

Cell state changes

The landscape is initially seeded with a predetermined number of cells at a defined location (with some stochastic variation). The cells then "move" stepwise across the landscape. At each time step, the cell's new position is determined by the local slope of the landscape, the local wind, and a probabilistic component (with random direction, but specified magnitude). These three sources of cell movement on the landscape represent internal regulation, external signals, and regulatory network noise, respectively. The simulation is then run to allow cells to reach new states, resulting in an animation representing the temporal evolution of the cells' states. In some cases, the magnitudes of either the "winds" or the stochastic variation are changed during the animation.

General principles and biological equivalents

A deep attractor basin represents a stable state in the regulatory network, typically mapping into a well-recognized cell (sub)type that would require a strong stimulus (wind) for that cell to escape and reach another state. Winds could represent, for example, antigen stimulation or the addition of cytokines in vitro, or an infection or other environmental change in vivo. The rate at which a cell can sample new states around its current state on the landscape is related to the stochastic variation and the winds and is inversely related to the height of the barriers between states. The portion of the landscape represented in each animation is typically inclined, so that many of the transitions are irreversible unless a strong wind is applied. This mimics the general trend for unidirectional differentiation.

The animations

Each animation is intended to stimulate discussion of a different concept. For each, we describe the *concept* being explored, the *biological examples* in which this concept might operate, the *implementation* of this concept in an animation, and the *conclusions and potential questions* that might be addressed by biological experimentation. Animations are provided in the Supporting Information, and frame captures from each movie are shown in Fig. 1.

Animation 1: Populations versus single cells

Concept

The differentiation of *cell populations* may be more predictable than the differentiation of *individual cells*. Due to the large degree of variability of expression of many cell products within a single population, different cells will not behave identically during differentiation, but the behavior of the cell population as a whole will be well-regulated if homeostasis—as represented by the size and depth of an attractor basin—is sufficiently strong.

Biological examples

This animation conceptually mimics the differentiation of naïve T cells into the moderately robust effector phenotypes, Th1 and Th2. Differentiation is assumed to proceed under the influence of weak Th1 and Th2 driving influences, which can result in a mixed Th1/Th2 population (J. Kobie and T. R. Mosmann, unpublished) and mixed, less dichotomous populations [91]. This example represents the elementary scenario in which a multipotent cell faces a fate decision, that is, it can differentiate into two alternative cell types.

Implementation

The landscape in Supporting Information Animation 1 and Fig. 1A has three attractor basins, an uphill naïve T-cell basin and two downhill effector (Th1 and Th2) basins. After a brief period demonstrating stability of the naïve state, a wind pushes cells out of the naïve basin, and the cells accumulate in the two effector states in equal proportions. The simulation is seeded with 500 cells that all have the same properties (except for stochastic variation). Two cells have been colored to allow them to be tracked in the animation.

Conclusions and potential questions

The animation results in the reproducible "differentiation" of the population of naïve T cells into the Th1 and Th2 phenotypes, in approximately equal proportions. Although the population results are consistent on repeated runs of the animation, the tracks of individual cells are highly variable, and the final destination of an individual cell cannot be predicted until late in the process. Thus, the behavior of cell populations is more predictable than the behavior of individual cells. This is consistent with our high-resolution analysis of flow cytometry data [75, 92], in which we can define populations with more precision than we can designate each individual cell.

Animation 2: Reticulate T-cell differentiation

Concept

As an extension of Supporting Information Animation 1, we propose that there are multiple semistable states (shallow basins) during the differentiation of T cells to a dichotomous outcome. Cells can take different paths through the intermediate states to reach the same final state. This process of reticulate differentiation may be conceptually analogous to reticulate evolution [93, 94] proposed, for example, for corals in response to shifting ocean currents [95].

Biological examples

This animation mimics naïve T cells differentiating to Th1 or Th2 effector states, but includes several semistable intermediate states. These could include the uncommitted Thpp [21, 77] and Tbet$^+$GATA3$^+$ [96] differentiation states that can both be intermediates on the Th1 and Th2 differentiation pathways.

Implementation

Supporting Information Animation 2 (Fig. 1B) shows the addition of shallow attractor basins and peaks within the broad valley, leading to the Th1 and Th2 effector states. Three of the basins were given the property of changing the color of any cell entering the basin (adding red, green, or blue to the existing cell color), thus indicating the partial history of each cell reaching the Th1 or Th2 states, for example, a yellow cell had passed through the green and red basins.

Conclusions and potential questions

Our speculative model suggests that cell differentiation may take alternative paths through quasi-discrete, metastable intermediate states, not necessarily passing through the same sequence of states. Can these states be identified? Longitudinal measurement of gene expression in single, living cells is required, which limits the approaches available for detecting intracellular molecules. Lineage tracing using bar coding [97], or fluorescent reporter constructs [98, 99], may provide insight into these possibilities [100]. Similar approaches have revealed intermediate states during induced stem cell reprogramming, including first and second waves driven by c-Myc/Klf4 and Oct4/Sox2/Klf4 transcription factors, respectively [101].

Animation 3: T-cell activation and re-randomization

Concept

Apparently stochastic initiation of cytokine expression by activated T cells is stable during a single stimulation cycle, but may be rerandomized within the population after return to the resting state [79]. The degree of rerandomization may vary depending on the time chosen for restimulation.

Biological examples

Th1 cells may express IL-2 in an apparently random pattern, whereas IFN-γ expression shows more continuity ("memory" for the previous state) [77]. This may be due to different kinetics of randomization.

Implementation

In Supporting Information Animation 3 (Fig. 1C), the resting state of a differentiated Th1 cell is depicted as a low basin on a landscape, with two uphill states representing IL-2$^+$ and IL-2$^-$ phenotypes. A strong wind (activation by antigen) pushes cells uphill toward the two states. Cells reaching each state are assigned a different color, then the stimulation "wind" is turned off, and cells fall back to the resting state. Cells are then reactivated without any further change in color.

Conclusions and potential questions

If restimulation is applied before the cells have returned fully to the resting phenotype basin, then some memory of the phenotype generated by the initial stimulation will be carried through to the second stimulation phenotypes (Supporting Information Animation 3A). If more time is allowed, the stochastic variability in the resting basin causes the second stimulation to produce cell populations with mixed histories (Supporting Information Animation 3B). Thus, the (re-)randomization of cytokine synthesis patterns may depend on elapsed time since the previous stimulation. This could be tested by mapping the extent of phenotypic conversion as a function of time, by sorting cytokine-positive and cytokine-negative cells and culturing for different times before restimulating and testing cytokine expression [79].

Animation 4: Single populations may have multi-modal distributions

Concept

If barriers between two states are low, that is, the stochastic variability readily causes intermixing of cells between the states, then the two states may normally exist as a single but multimodal population (overlapping "peaks") in which all cells are in short-term equilibrium with both substates.

Biological examples

Supporting Information Animation 4 mimics the variable expression of cytokines in an otherwise uniform population [78, 82, 102]. Cytokine + and − states may be due to the "capture" of two

preexisting states, that is, the starting population may exist in a bimodal state, and activation freezes the cells in these two half states. In principle, if a process can be regulated by a single mRNA molecule (e.g., for a transcription factor regulating several downstream genes), if the rates of synthesis and degradation of that mRNA result in approximately 50% probability of a cell expressing that mRNA at any one time, and the rate of $+/-$ conversion is significantly longer than rates of protein synthesis and degradation, then the population can be bimodal.

Implementation

Two overlapping basins are shown, with two starting cell populations in different colors in each basin. The simulation is allowed to run with no external wind, that is, cells move only according to the landscape and stochastic variability. Figure 1D shows that the total population always remains bimodal, while the two starting populations approach equilibrium after 1000 cycles of the simulation.

Conclusions and potential questions

Although the cells are concentrated mainly in the two basins, these are in equilibrium. If a population is defined as a group of cells with freely interchangeable properties in the absence of external influences, then even a single population may display a multimodal distribution if there are distinct sets of possible properties (i.e., partially separated attractors). As in Supporting Information Animation 2, fate mapping by bar coding or fluorescent reporter constructs may help to identify such populations.

Animation 5: Robustness and plasticity of T-cell effector phenotypes

Concept

The landscape model suggests that both robust, difficult-to-alter T-cell phenotypes and flexible T cells with further differentiation potential may be represented just by quantitative changes in the shape and relative depth of the attractor basins.

Biological examples

Th1 and Th2 are robust phenotypes that are stable under many (but not all) circumstances. In contrast, Thpp cells can easily be induced to differentiate into either Th1 or Th2 phenotypes, Treg cells can differentiate into Th17-like cells, and Th17 cells can differentiate into Th1-like cells [54, 59, 60].

Implementation

The landscape comprises an initial uphill basin representing naïve cells, and four downhill attractors representing Th1, Th2, Treg, and Th17. The Treg and Th17 basins are at an intermediate level, and have a low barrier between these two states, whereas the Th1 and Th2 basins are deep, further downhill, and well-separated from the other basins. Cells start in the naïve basin (Supporting Information Animation 5A, to populate all four differentiation states) or in the Th1 or Treg basins (Supporting Information Animation 5B and Fig. 1E).

Conclusions and potential questions

Although naive cells can become any one of the four effector phenotypes, once a cell has differentiated into a Th1 or Th2 cell, it is difficult to induce further differentiation because of the deep Th1 and Th2 attractor basins. Th17 and Treg attractors are not as strongly separated, so further differentiation is possible with moderate external stimuli. Thus, the difference between stable and plastic cell phenotypes may be a matter of degree, that is, more stable phenotypes may simply be cells that need a stronger push, such as may occur in vivo during strong responses to infection [62, 64]. Such redifferentiation may occur directly (transdifferentiation), or by partial dedifferentiation followed by redifferentiation [103]. The landscape in Supporting Information Animation 5 also suggests that Th17 or Treg cells, under the right conditions, could be pushed into the downhill Th1 or Th2 basins. This is explored in more detail in the next animation.

Animation 6: Parallel or convergent differentiation of T cells

Concept

Cells that acquire similar properties may represent convergence to the same state, or may be cells that share some properties but are distinct in others (which may not be measured). Therefore, if the model includes information on all variables, similar but nonidentical states will occupy different attractor basins, but if one or more distinctive (stratifying) variables are not measured, this may result in "hidden" dissimilarity.

Biological examples

This animation mimics the induction of IFN-γ production by human Th17 cells—these "Th1-like" cells still maintain differences from normal Th1 cells [104]. Similarly, Th1 cells can be induced to produce IL-4 during a parasite response in vivo [62]—these may be true Th2 cells, or the Th1-derived cells may maintain some of the initial Th1 regulatory pathways.

Implementation

Convergent differentiation is illustrated using two landscapes in which there is an initial separation into two valleys leading to Th1 and Th17 states. In the first landscape, a wind then pushes the Th17 cells further down the valley to fully converge with Th1 cells (Supporting Information Animation 6A and Fig. 1F left). The second landscape illustrates parallel differentiation without true convergence, as Th17 cells are pushed to an attractor basin close to the Th1 state by the same wind, but never reach the actual Th1 state (Supporting Information Animation 6B and Fig. 1F right) because of an intervening ridge.

Conclusions and potential questions

This model illustrates the principle that the sharing of some key (monitored) characteristics does not mean that two cell states are identical. Therefore, it is important to check as many markers as possible to capture the discriminatory variables, and particularly to test cell behavior experimentally, before concluding that cells have truly converged on an identical state. Th1-like cells may be derived from Th17 cells by parallel differentiation [104], whereas conversions from Th1-like to Th2-like cells, or from Treg cells to Th17-like cells may need further examination [62, 105].

Animation 7: Cell interaction – positive feedback

Concept

Supporting Information Animations 1–6 assumed that cells differentiate as independent units only under the influence of landscape, winds, and stochastic fluctuations. However, cell–cell interactions can influence differentiation rate and direction in both positive and negative regulatory loops. A more realistic model therefore needs to incorporate this principle.

Biological examples

This example mimics the differentiation of Th2 cells, which produce IL-4, which strongly enhances differentiation of naïve cells into more Th2 cells. Similarly, IFN-γ and IL-17 participate in positive feedback loops for Th1 and Th17 cells, respectively.

Implementation

Supporting Information Animation 7A is set up as in Supporting Information Animation 1, with the addition of a small "wind" pushing cells toward the Th1 state (cells turn green on arrival). However, cells arriving in the Th2 attractor basin (red in the animation) acquire the property of contributing to a "wind'" pushing

cells in the Th2 direction (Fig. 1G). Note that this is a slightly different use of the "wind," which in all other animations represents only exogenously applied signals. The endogenous and exogenous "winds" are depicted by colored arrows in Supporting Information Animation 7B. (In an alternative model, feedback could be captured by deforming the landscape: for example, cells that arrive in the Th2 attractor will deepen and enlarge its basin of attraction.)

Conclusions and potential questions

Naïve cells differentiate predominantly into Th1 cells at first, but as mature Th2 cells accumulate, further differentiation becomes more biased toward the Th2 pathway. The model also suggests that positive feedback influences not only the overall outcome, but may also alter the proportion of cells in intermediate states.

Animation 8: Variability increased by external forces

Concept

Instead of a wind, differentiation may also be initiated by increasing stochastic variation in a population, overcoming the barriers around a relatively stable state. This could be represented as "heat" that increases the stochastic fluctuations of cell states within a basin. For a given attractor basin, there will be a level of stochastic variation below which all the cells will remain in that attractor state, and above which cells occasionally "spill over" out of the basin, that is, there is a minimum "escape velocity" required for leaving an attractor.

Biological examples

The apparently stochastic variability of gene expression within a population can be regulated [106], with positive and negative feedback generally increasing or reducing the noisy fluctuations, respectively. Probabilistic gene expression is well-described in the immune system [107], but we are not aware of *regulation* of this variability in immune cells. However, an example in another cell type is that Wnt signaling may regulate the variability of gene expression of key regulators in neuroblast development, thereby controlling the probability that a cell exits the multipotent state and enters an adjacent attractor—representing differentiation [106, 108].

Implementation

In Supporting Information Animation 8, differentiation is initiated not by a "wind" but rather by increasing stochastic variation, or "heat," so that cells occasionally acquire enough energy to escape

the naïve cell basin (Fig. 1H). The shape of the landscape dictates that escaping cells mainly flow toward the effector attractors.

Conclusions and potential questions

Variability of expression and modification of gene products within an otherwise uniform population may thus be an important regulatory mechanism for cell differentiation, instead of representing inconsequential noise. This would provide an alternative mechanism for initiating differentiation by expanding the range of states that could be sampled by naïve cells. Once initiated, the overall landscape dominantly directs further differentiation. Are there examples of external regulation of gene product expression fluctuations in T cells?

Animation 9: Chronic, weak influences may induce long-term effects

Concept

If stochastic variation barely reaches an escape threshold, a very weak "wind" may slowly transfer cells to a "downhill" state that will react differently when stimulated.

Biological examples

Supporting Information Animation 9 (Fig. 1I) mimics chronic inflammation in aging (inflammaging) [109], which may alter the long-term responsiveness of T-cell populations. Other chronic inflammatory states include chronic infections and autoimmunity.

Implementation

A weak "wind" (chronic inflammation) slowly pushes naïve cells from one low-lying attractor basin to another even lower basin, with the rate being controlled by the joint effect of the "wind" and the stochastic variation that provides rare outlier cells that escape the initial attractor. If antigen stimulation (an exogenous "wind" that blows the cells uphill) occurs early, most cells are activated from the normal population, whereas later stimulation will act also on cells that have accumulated in the "inflammaged naïve" attractor basin (orange cells in the animation).

Conclusions and potential questions

In chronic inflammatory conditions, very slight effects may slowly alter populations if cells reach a transfer threshold at a very low rate. However, once the cells have transitioned to the inflammation-induced state, "uphill" reversion to normal may be more difficult. This simulation also emphasizes the importance of the interplay between the magnitudes of four parameters: wind (representing external stimuli), depth of attractor basin (representing the stability or robustness of the regulatory network of a particular state), stochastic variability of protein expression and modification, and time (age). Note that the effect of aging could be either to provide a small wind (Supporting Information Animation 9) or to increase variability, as in Supporting Information Animation 8. While Supporting Information Animation 9 is intended to depict the alteration of naïve cells due to chronic inflammation, similar animations could be designed to illustrate slow conversion of memory cells to an "inflammaged" phenotype.

Further animation possibilities

Proliferation and death

Differentiation of CD4$^+$ T cells is normally accompanied by extensive proliferation. This could be modeled in the LAVA animations by assigning a probability of division to individual cells in certain states, with some interesting consequences. Daughter cells could stochastically end up in different attractors. The outcome may also be affected by the phenomenon of asymmetric division, in which T cells can become polarized by interaction with an antigen-presenting cell, leading to different properties of the two daughter cells [110–112]. Undirected, random asymmetric division adds stochasticity, whereas directed asymmetric division could be modeled in LAVA as reciprocal "jumps" of the two daughter cells into two distinct (polarized) states. A third point of interest is that selectively increased proliferation of cells in a particular state may appear as increased differentiation toward that state. Although proliferation could be represented in an additional dimension (axis) of the state space, this is difficult because the animations become crowded in higher dimensional spaces, given that the landscape intuition exists only in the 3D animation. Analogous to the effect of selective proliferation, selective death in certain states could also influence the proportion of cells in the final effector basins and thus also contribute to selective differentiation. This could also be modeled in LAVA.

Closing comments

In developing these animations, we were struck by the importance of stochastic variation for obtaining a satisfactorily plausible outcome. It has been proposed that variation within cell populations is not just an irrelevant property of the system, but has real value in smoothing dose–response curves, providing more graded, regulatable responses [76, 113], and controlling access to further differentiation states [90]. The example of regulated variability in stem cells versus more differentiated cells [106] opens up the possibility that this is a more general mechanism, and that regulation of variability, along with the constraints of the landscape topography, could fine-tune stability and flexibility of defined T-cell

types. Mechanisms that generate variability within a single state (i.e., in one basin) are likely to cause variability in phenotypic behavior. Interestingly, flow cytometry shows us that many proteins are expressed over a substantial range (more than tenfold), far exceeding technical noise (approximately two- to threefold [90]) and that for each protein, this variation is apparently random relative to most other proteins. Is the consistency of this range a consequence of regulated variability? What types of mechanism regulate variability in lymphocytes?

In flow cytometry data, cell populations appear as diffuse clouds that often overlap with other populations even in high dimensions [75, 92]. This single-cell resolution information offers a more accurate picture of the differences between the states than bulk biochemical measurements on populations because the diversity of the cells within a recognized phenotype is an important property of the population [90, 114, 115]. It is tempting to draw parallels between the "clouds" in flow cytometry and the attractor basins populated by heterogeneous cells in the landscape model. Although attractive, this has to be interpreted cautiously. While the landscape and attractor basins are more than just a metaphor, and can be reduced to first principles of the dynamics of regulatory networks that dictate the stability of cell states [3], specific quantitative details of all regulatory pathways, necessary for quantitatively accurate landscape models, are still lacking. Notwithstanding, the landscape visualizations, informed by general principles of how molecular networks dictate cell phenotypic states, suggest that a probabilistic approach to T-cell differentiation and state stability may capture essential biological properties, and may make us more comfortable with heterogeneous cell populations in which only a proportion of the cells show a particular behavior. The probabilistic description may not be as intuitive, but may be a more accurate description of cellular behavior.

In closing, we would like to emphasize that the visualizations we present are intended to serve as a conceptual framework to help thinking about a complex high-dimensional and probabilistic T-cell differentiation process, and to provoke discussions that are not easily conducted by talking in the abstract. The LAVA animations capture the generic features of regulatory systems: probabilistic processes constrained by deterministic regulatory interactions. We hope that these animations will bring several concepts into the 3D realm with which we are most comfortable, thus facilitating discussion.

LAVA availability

The LAVA program (MATLAB) is available on request at http://www.ece.rochester.edu/projects/siplab/Software/LAVA.html

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