

current daily intake of small molecule anti-retrovirals (Caskey et al., 2015; Ledgerwood et al., 2015). Additionally, bNAbs typically retain important Fc effector functions, which might play a key role in activity against infected cells (Boesch et al., 2015), which is of potential import in cure strategies. Finally, antibody delivery for prophylaxis or treatment holds particular promise if, like N6, the antibody is highly potent, thereby reducing required dose and, consequently, unit costs.

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## Expression Diversity Adds Richness to T Cell Populations

Dean Franckaert<sup>1,2</sup> and Adrian Liston<sup>1,2,\*</sup>

<sup>1</sup>Translational Immunology Laboratory, VIB, 3000 Leuven, Belgium

<sup>2</sup>Department of Microbiology and Immunology, KU Leuven - University of Leuven, 3000 Leuven, Belgium

\*Correspondence: [adrian.liston@vib.be](mailto:adrian.liston@vib.be)

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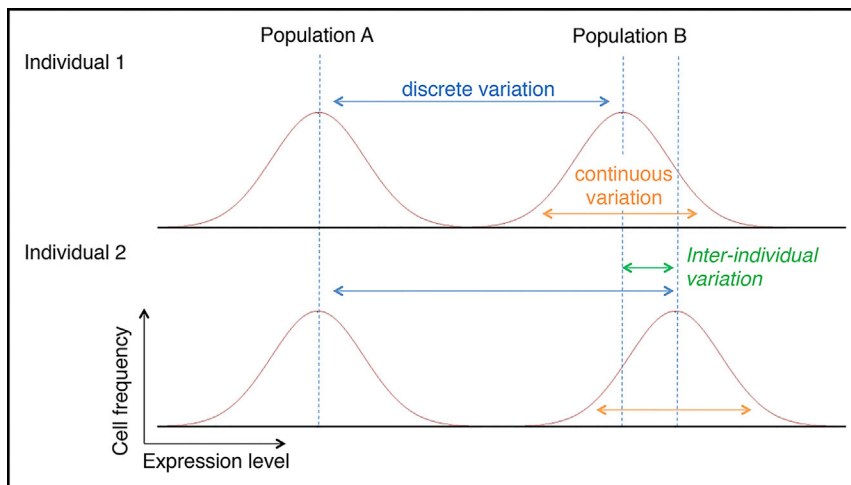
Variation in protein expression is a feature of all cell populations. Using T cell subsets as a proof-of-concept, Lu et al. (2016) develop a framework for dissecting out the contributors to this cell-to-cell expression variation from high-parameter flow cytometry studies.

Subsetting, one of the favorite pastimes of T cell immunologists, involves identifying clusters of cells with a distinct function and seemingly homogeneous expression profile. However, even within the most tightly defined populations, substantial heterogeneity in gene expression exists at the cell-to-cell level. This diversity arises due to stochastic processes, differential environmental input, and intrinsic differences in cellular history. Typically such heterogeneity is dismissed as noise; however, growing evidence indicates that the cell-to-cell diversity within “homogeneous” populations can contribute to divergent functional outcomes (Antebi et al., 2013; Newell et al., 2012). A report by Tsang and colleagues in this issue of *Immunity* develops a framework for analysis of cell-to-cell expression variation from high-parameter flow cy-

tometry datasets. Using this approach, the researchers demonstrate that the cell-to-cell expression variation within discrete subsets varies from individual to individual, is stable over time within a given individual, and is controlled in part by age and genetic background (Lu et al., 2016).

Expression variation exists in multiple categories (Figure 1). Discrete variation is observed between subsets of cells, each centered on a different median expression point; an example is the on-off expression distinction of major subset markers. The median expression of a subset can also vary from individual to individual, such as occurs via the influence of expression quantitative trait loci (Raj et al., 2014; Ye et al., 2014). A third category of variation, studied in much less detail, is the continuous

expression variation within each defined population. Tsang and colleagues studied this problem in 61 healthy human subjects, assessed at three time-points, by examining the continuous cell-to-cell expression variation present for 14 markers within 28 tightly-defined T cell subsets. They found that profound differences in expression heterogeneity within T cell subsets for different proteins. The variation in lineage markers such as CD3 and CD45 was generally stable across cell populations and time within a given subject, whereas expression variation in proteins that demark cellular states such as activation (e.g., HLA-DR) was unstable over time, likely reflecting the greater role of environmental input. Notably, the cell-to-cell variation in expression differed between subsets, indicating a diversity of



**Figure 1. Categories of Cell-to-Cell Expression Variation**

Classical flow cytometry histograms present the frequency of cells as a function of the expression of a given marker. A simple expression pattern might consist of a population that is “negative” for the marker (population A) and a population that is “positive” (population B). The expression difference between cells in these two populations can be considered discrete variation. Even within a single population, cell-to-cell expression variation exists (continuous variation), with some cells sitting above or below the median expression value of the population. A third layer of variability is added when multiple individuals are assessed, where the expression pattern of a single population (e.g., population B) is altered between individuals, either in terms of median expression or in terms of the variance from the median.

regulatory mechanisms at play for even a single protein.

At least some of the differences in the cell-to-cell expression variation between different proteins are likely to have biological significance. When individuals of different ages were assessed for cell-to-cell expression variation, significant age-dependent changes were observed (Lu et al., 2016). For example, assessment of CCR7 expression of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T (Treg) cells, found higher levels of cell-to-cell variation in older persons when compared to young adults. As CCR7 is needed for lymphocyte migration, increased heterogeneity could influence the tissue homing behavior of Treg cells, altering the local balance between tolerance and immunity. It is possible that such differences in cell-to-cell expression variation could contribute to the functional degeneration of immunity observed in older persons.

Genetic control over cell-to-cell expression variation was also observed. Lu et al. (2016) found ten unique interactions between cell-to-cell expression variation and genetic polymorphisms linked to (auto)immune conditions such as multiple sclerosis, asthma, rheuma-

toid arthritis, and Crohn’s disease. One striking example was the association of rs1588265, an intronic SNP in *PDE4D*, with the degree of cell-to-cell expression variation in HLA-DR on CD8 T cell subsets. The minor allele of rs1588265, protective against asthma, was associated with increased variation in HLA-DR expression (i.e., a broader spread of low- to high-expressing cells). Critically, this association could not have been picked up using classical approaches: the different alleles of *PDE4D* did not alter the mean expression of HLA-DR, impacting only on the cell-to-cell variation around the mean. While this association does not demonstrate that the minor allele of *PDE4D* confers protection to asthma through increasing the variability of HLA-DR expression, it does suggest that the degree of variation, as well as mean expression, might alter disease susceptibility or progression.

One of the major questions arising from this study is the relationship between cell-to-cell expression variation and T cell receptor (TCR) clonality. Due to genomic rearrangement, the TCR is distinct at the sequence level on a cell-to-cell basis between each newly generated T cell.

Because TCR engagement is obligatory for T cell development, the differential affinity of TCR for self-antigen-MHC complexes can leave its imprint on “tuning” molecules, adjusting baseline signaling to a common tonic level (Davis et al., 2007). To what extent are the properties of cell-to-cell expression variation directed by TCR sequence differences? Is the observed variation inherited in a clonal manner, such that competition between activated T cells is driven by both TCR sequence difference and cell-to-cell expression variation in key molecules? It is distinctly possible that variation in the expression of key TCR signaling molecules is clonally inherited and enhances the functional diversity of T cells. Under this scenario, additional heterogeneity might increase the chance of a T cell undergoing productive stimulation, elevating the utility during infection. The corollary is also notable—increased expression diversity could elevate the risk of an inappropriate reaction. These same arguments have been made for TCR sequence diversity, where the reduction of thymic output with age might be an evolved response to the rising cost-benefit ratio of TCR diversity with age (Dowling and Hodgkin, 2009).

Beyond these findings, the current study provides a resource for other investigators to identify cell-to-cell variation changes that might have biological significance. These results are limited to the scope of markers used in the original study, and it is unclear to what extent the observed properties will be extended to other proteins. Because markers are a non-random set of proteins, the original selection might have resulted in a variability profile that is relatively unrepresentative. We also lack validation of these measurements using independent antibody clones and dyes, or an independent technology such as Cytometry by Time of Flight (CyTOF), and it is likely that some differences represent technical issues rather than biological properties. The major contribution of Tsang and colleagues in this paper is to open up the field of cell-to-cell variation for further investigation, providing a framework for analysis and the software tools required for larger scale comprehensive assessment of the physiological impact of cell-to-cell expression variation.

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