Figuring Fact from Fiction: Unbiased Polling of Memory T Cells

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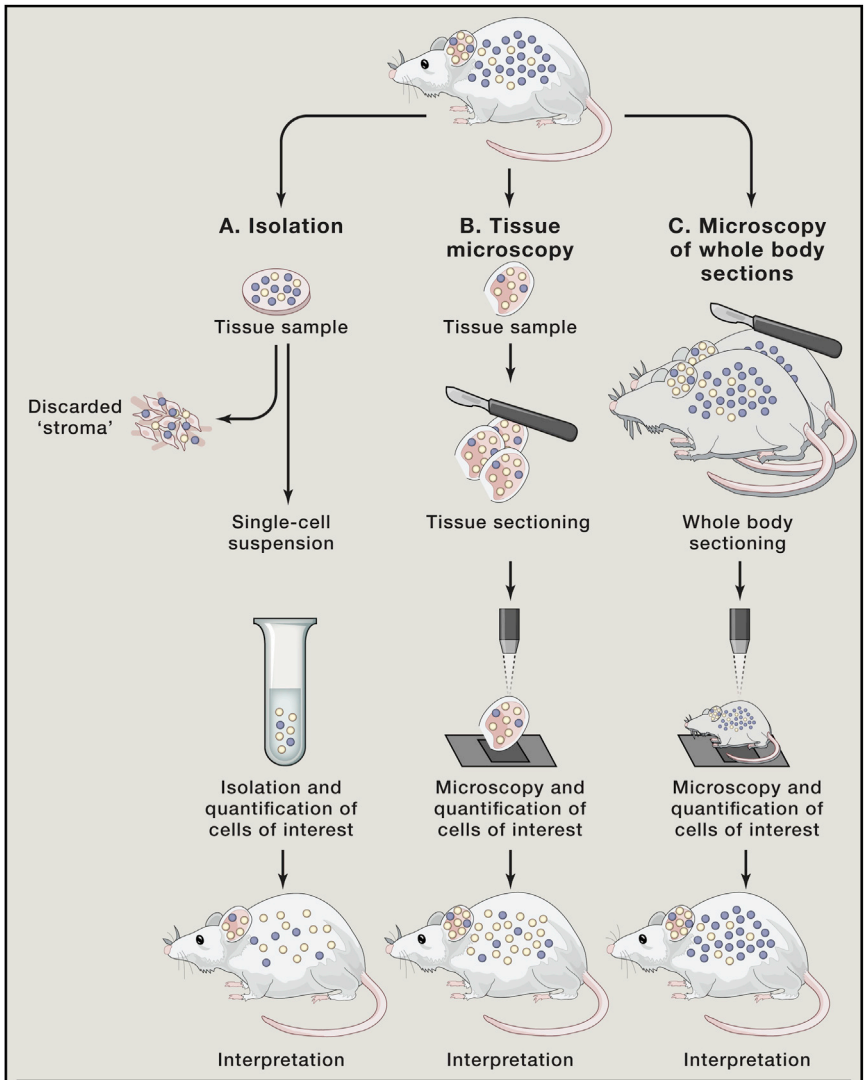
Immunization generates several memory T cell subsets that differ in their migratory properties, anatomic distribution, and, hence, accessibility to investigation. In this issue, Steinert et al. demonstrate that what was believed to be a minor memory cell subset in peripheral tissues has been dramatically underestimated. Thus, current models of protective immunity require revision.

In 1936, *Literary Digest*, a political magazine, surveyed a quarter of the U.S. voting population and predicted that Senator Alfred Landon would capture 55% of the vote and defeat the incumbent Franklin D. Roosevelt. On Election Day, Roosevelt soundly defeated Landon with 61% of the vote, the largest margin of victory in history at the time. How could the magazine's polling have been this embarrassingly misleading? The answer lay in the methodology that was used, particularly the inherently biased sampling of respondents whose names could be easily obtained from phone directories and automobile registration records, a group that was not representative of contemporary U.S. voters (Squire, 1988). This kind of bias easily creeps into political polls, and careful measures are now being taken to avoid such pitfalls.

In science, however, we sometimes forget that the methodologies we use can similarly skew what appear to be objective outcomes. In this issue, Steinert et al. (2015) provide a telling example of how a widely used analytical approach in cellular immunology has distorted the field's concepts of immune surveillance by memory T cells. The authors demonstrate that the traditional approach relied on data extrapolation from apparently non-representative samples and the use of unreliable surrogate markers for functional definitions of cellular subsets.

Immune challenges, such as infections or vaccination, result in the activation (also called "priming") of naive T lymphocytes in secondary lymphoid organs (SLOs). Some of the activated T cells differentiate into so-called memory cells, which have the capacity to persist for many years after the original challenge has been cleared. Importantly, memory cells provide enhanced protection against re-infection with the same pathogen. Memory T cells are usually classified into three distinct subsets based on each subset's unique migratory behavior (Mueller et al., 2013; Sallusto et al., 1999). Central memory T cells (T_{CM}) circulate through blood and SLOs, including the lymph nodes, which collect lymph fluid from the body's peripheral tissues. Effector memory T cells (T_{EM}) lack lymph node homing capacity; T_{EM} are found in blood and spleen and were widely assumed to also survey non-lymphoid tissues. More recently, a third memory T cell population was identified: the tissue resident memory T cells (T_{RM}). T_{RM} arise soon after priming from activated effector cells that seed peripheral tissues. Unlike T_{EM}, which have been thought to visit such tissues transiently, T_{RM} are largely sessile and do not circulate. Recent studies revealed that, at least in some settings, T_{RM} are more effective at protecting non-lymphoid tissues from pathogens than the migratory T_{CM} and T_{EM} (Mackay et al., 2012). This posed an apparent conundrum because T_{RM} were believed to be sparse and vastly outnumbered by their neighboring parenchymal cells. Since T cells must directly touch every infected cell that they are meant to protect, how could the rare T_{RM} be so effective at protecting the abundant somatic cells from invading pathogens?

An early glimpse of the overall distribution of the memory T cell repertoire in immunized mice was provided in 2001 by two classical studies that showed that most memory cells reside in peripheral tissues and not in SLOs (Masopust et al., 2001; Reinhardt et al., 2001). One of these studies tracked CD4 memory cells by immunohistochemical analysis of whole-body sections of immunized mice (Reinhardt et al., 2001), a tour-de-force strategy that yields unbiased results but is technically highly demanding. Thus, more recent studies in the field have resorted to quantifying memory T cells in single-cell suspensions of tissues that were freshly harvested from immunized mice (Figure 1). To distinguish between the different memory cell subsets, researchers stain the recovered T cells with antibodies to lymph node homing receptors (expressed on T_{CM}, not T_{EM} or T_{RM}) and to two surface markers, CD69 and CD103, which were thought to be diagnostic for T_{RM}. Several studies have distinguished between extra- and intravascular memory cells by intravenously injecting an antibody to a common T cell surface moiety (e.g., CD45) coupled to a large fluorophore, such as phycoerythrin, a few minutes prior to sacrificing the animal. The injected antibody remains confined to the vessel lumen during this brief time interval, so it stains selectively the intravascular subset (Anderson et al., 2014). The extravascular T cells, which remain unstained, are composed of non-migratory T_{RM} and additional memory cells that access peripheral tissues sporadically from the blood and eventually depart via the draining lymphatics (Mackay et al., 1988). The latter have



Technical difficulty	Low	Low	High
Throughput	High	Moderate	Low
Multi-parameter analysis	High	Limited	Limited
Quantitative data accuracy	High	Limited	Limited
Potential for sampling error or bias	High	Moderate	Low

Figure 1. A Comparison of Analytical Methods to Quantify Memory T Cells in Immunized Mice

Colored spheres represent memory T cells that are non-randomly dispersed throughout the body. Different colors symbolize different subsets of memory T cells (only two subsets are shown for simplicity). (**A**) In the most common approach in the field, a tissue sample is enzymatically digested and mechanically dissociated to generate a single-cell suspension, while indigestible tissue stroma is discarded. In this approach, T cell isolation is often incomplete, isolation efficiency can vary between T cell subsets, and information regarding the spatial localization of the T cells within the tissue and body is not preserved. (**B**) Tissue samples are sectioned and analyzed by immunostaining and quantitative microscopy. Data from the analyzed region/tissue is extrapolated to the whole organ and even the whole mouse. Information regarding the density and spatial distribution of T cell subsets within the analyzed sample is well conserved, but results may not necessarily be representative of the whole mouse. (**C**) Analysis of whole-body sections by microscopy can provide information regarding the spatial distribution of T cell subsets within an entire animal; however, the approach is technically very demanding.

long been assumed to be recruited from the T_{EM} subset, although experimental evidence has largely been lacking.

These standard procedures for memory cell isolation have been relying on two assumptions: (1) T cell isolation from tissue-derived cell suspensions is efficient and yields every memory subset without bias, and (2) the presence and identity of T_{RM} is faithfully reported by CD69 and/or CD103 expression combined with lack of intravascular staining. In this issue, Steinert et al. test both assumptions by comparing the frequency and phenotype of each memory subset recovered from traditional tissue suspensions with results obtained using exacting quantitative microscopy of immunostained tissue sections (Figure 1). The results are unexpected. The number of T_{RM} that are found in sections of some peripheral tissues, such as the female reproductive tract (FRT), is much larger (by as much as 60-fold) than the number of T_{RM} that can be recovered from single-cell preparations of the same tissues. This discrepancy reflects a dramatic loss of T cells during tissue processing, whereby many cells are presumably either killed or discarded with indigestible tissue stroma. T cell loss disproportionately affects the recovery of T_{RM}, resulting in over-representation of other memory subsets, particularly those in the intravascular compartment. Furthermore, when the two analytical techniques are applied to other tissues, such as spleen and lymph nodes, both approaches yield comparable numbers of memory cells. These findings imply that the standard model of peripheral T cell memory, which has been largely based on analyses of tissue suspensions, not only underestimates the overall size of the memory pool, but also is based on a severely skewed perception of subset abundance both between different

anatomic regions and within any given tissue.

Steinert et al. also interrogate the second assumption: that T_{RM} faithfully express CD69 and/or CD103 and are not accessible to intravascular antibody. Using parabiotic pairs of congenic mice, which were surgically joined to establish a shared blood circulation, the authors discover that a sizeable fraction of T_{RM} express neither CD69 nor CD103, and some T_{RM}, especially in the kidney and liver, actually appear to reside within the intravascular space.

These findings have implications for how immunologists think about T cell surveillance of tissues, particularly with regard to T_{RM}. For example, in the FRT, isolation-based methods had estimated that there is one T_{RM} for every ~20,000 nucleated cells, while tissue microscopy performed by Steinert et al. reveals that there is one T_{RM} for every ~300 nucleated cells. Assuming that T_{RM} within the FRT scan cells at a similar rate to those in the skin (Ariotti et al., 2012), isolation-based methods project that T_{RM} would require ~1 month to scan every cell in the FRT. In contrast, the tissue microscopy data imply that T_{RM} scan the FRT in its entirety within ~12 hr, an estimate that is much more consistent with the reported effectiveness of T_{RM} to protect non-lymphoid tissues (Mackay et al., 2012).

Steinert and colleagues thus provide a much-needed reality check for immunologists. Their findings will have to be taken into account when evaluating immune responses to vaccines and pathogens, and it will be important to determine their impact on our understanding of allergic and autoimmune diseases, as well as immuno-oncology.

Even though 80 years have passed since the *Literary Digest* fiasco, this study provides a stern reminder that sample bias is not a fiction of the past but remains to this day a fact to be reckoned with—by scientists and voters alike.

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Shedding Light on Glioma Growth

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Cancer is known for opportunistically utilizing resources from its surroundings for its own growth and survival. In this issue of *Cell*, Venkatesh et al. demonstrate that this also occurs in the brain, identifying neuronal activity-induced secretion of neuroligin-3 as a novel mechanism promoting glioma proliferation.

Cancer is notorious for hijacking normal biological processes to promote tumor cell survival, migration, and proliferation. Cancer cells release angiogenic factors that promote blood vessel formation to support their own survival and upregulate molecules normally expressed by healthy cells to evade immune detection. In their recent study, Venkatesh et al. (2015) reveal that cancer cells also take advantage of neuronal activity, the most essential aspect of brain function, in order to proliferate. The authors demonstrate that optogenetic stimulation of neurons can promote the growth of human high-grade gliomas (HGGs) by inducing the secretion of mitogenic factors.

This study was initiated following the discovery that neuronal activity stimulates the proliferation of oligodendrocyte precursor cells (OPCs) and neuronal precursor cells (NPCs) in vivo (Gibson et al., 2014), cells that can give rise to gliomas (Cuddapah et al., 2014). Both studies utilized optogenetic strategies to increase neuronal activity by stimulating channelrhodopsin-expressing neurons with blue light (Figure 1A). This approach enables the activation of subsets of neurons in defined circuits in a physiological manner and allows for comparisons between different circuits or regions from within the same brain. Importantly, this method