



Detection of Antigen-Specific T Cell Lineages and Effector Functions Based on Secretory Signature

Greg A. Kirchenbaum, Jodi Hanson, Diana R. Roen, Paul V. Lehmann*

Cellular Technology Limited (CTL) Shaker Heights, OH, 44122-5350, USA

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*Correspondence:

Dr. Paul V. Lehmann, Cellular Technology Limited (CTL), 20521 Chagrin Boulevard, Shaker Heights, Cleveland, OH 44122-5350, USA; Telephone No: +1-216-791-5084; Email: paul.lehmann@immunospot.com.

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ABSTRACT

T cells not only protect us from infectious diseases and cancer, but are also involved in transplant rejection, autoimmune diseases, and allergies. Each of these immunologic processes share a common link in which antigen-specific T cells undergo expansion, with some of the resulting progeny differentiating into memory cells. Memory T cells belong to several distinct lineages, and sub-lineages, that fundamentally differ in their effector functions and capacity to mediate a protective or pathological immune response. In this mini-review, we outline how such memory T cell subpopulations can readily be identified on the basis of their secretory signature using a multi-color ImmunoSpot® assay.

The human immune system is composed of both an innate and adaptive arm (Figure 1). Innate cells, such as macrophages and neutrophils, serve as the first line of defense against foreign invaders by recognizing common features of pathogens using a limited number of conserved pattern recognition receptors^{1,2}. While the innate arm of the immune system conveys protection against most environmental microorganisms, it is unable to recognize unique features of pathogens, possesses limited effector functions, and is unable to generate immunological memory¹.

The adaptive immune system, consisting of B and T lymphocytes, bridges this gap. B cells are responsible for the production of antibody (immunoglobulin), while T cells engage in direct cell-mediated immunity¹. B and T cells are fundamentally different from cells of the innate immune system in as much as they are not limited to recognition of common pathogen features. Instead, individual B or T cells bear highly variable and unique antigen receptors, termed B cell or T cell receptors, respectively³. While any B or T cell is reactive with only a limited number of antigens, collectively all B and T cells in the body can recognize essentially any self- or foreign-antigen. Both B and T cells bestow the adaptive immune system with immunologic memory, that is, the capacity to respond to an antigenic insult with increased speed and vigor upon a secondary encounter⁴.

The frequency of B or T cells specific for any given antigen is, with few exceptions, very low^{5, 6}. The task of detecting them is therefore akin to finding a "needle in a haystack". Following a viral or bacterial infection, or as the consequence of vaccination, very rare antigen-specific naive B and T cells ($\ll 1$ in 10^6 white blood cells, also called peripheral blood mononuclear cells, PBMC) engage in vigorous proliferation, and their frequencies increase sharply⁷⁻¹¹. Within 10 days of initiating an immune response, antigen-specific B and T cells can account for up to 1%, but more typically 0.1%, of

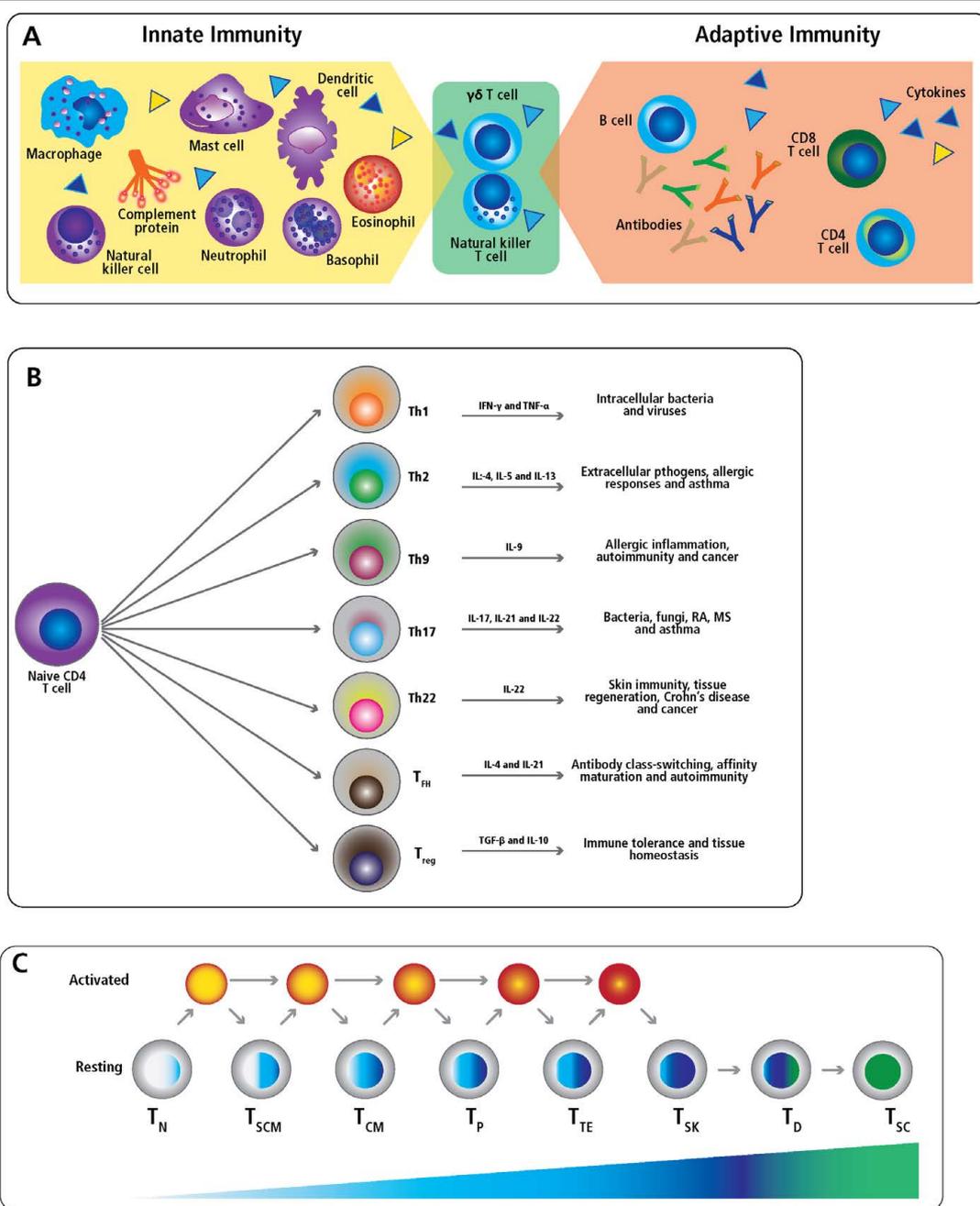


Figure 1: Components of the innate and adaptive immune system. (A) The human immune system is composed of both the innate and adaptive arms. Cells of the innate immune system are indicated in the yellow shaded region, along with innate effector molecules such as cytokines (triangles) and complement. In the green shaded region, specialized T cell populations with innate-like properties, which serve to bridge the gap between innate and adaptive immunity, are highlighted. Components of the adaptive immune system including B cells, secreted antibody, T cells and cytokines are indicated in the orange shaded region. (B) Schematic of CD4⁺ T cell subsets, including their principal cytokine signature and association with specific immune responses. (C) A model of progressive CD8⁺ T cell differentiation is illustrated. The CD8⁺ T cell subpopulations are, as specified in the text: naïve T cells (T_N); stem cell-like memory T cells (T_{SCM}); central memory T cells (T_{CM}); polyfunctional memory T cells (T_P); Terminal effector T cells (T_{TE}); silent killer effector cells (T_{SK}); dysfunctional T cells (T_D); and senescent T cells (T_{SC}). Blue shading of resting T cell populations denotes progressive differentiation and loss of effector functionality. Prolonged activation of CD8⁺ T cells leading to progressive differentiation, including bypass of developmental intermediates, and transition toward loss of proliferative capacity is depicted using yellow to red shading. Green shading of T_D and T_{SC} subpopulations denotes T cell exhaustion and loss of effector function.

all lymphocytes in PBMC¹²⁻¹⁴. In the subsequent weeks, irrespective of whether the antigen is completely cleared or chronically persists, these antigen-specific populations contract and their frequencies decline¹⁵. Typically, antigen-

Table 1. Assay platforms for assessment of T cell immunity. Approaches for evaluating T cell immunity, including cytokine production and proliferation, along with advantages and drawbacks are listed.

Assay	Advantages	Drawbacks
ELISPOT / FluoroSpot	Low cell input requirement/assay miniaturization Single-cell resolution/low limit of detection Multi-analyte detection/multiplexing High-throughput scalability Functional phenotyping Non-destructive/cell re-utilization	No surface phenotype Limitation in multiplexing Assay interference due to cytokine capture Compatible antibody pair requirements No direct measurement of cell proliferation
Tetramer / Multimers	Direct identification/quantification of peptide-specific T cell populations Compatible with multi-parametric surface phenotyping Single-cell resolution Parallel assessment of independent T cell populations using multiple detection probes	Assay sensitivity is dependent on T cell receptor avidity and expression level Restricted to assessment of predefined T cell populations based on reagent availability Limited to donors with specific HLA genotypes
ELISA	Simple Wide range of commercially available kits High-throughput scalability/batch analysis	No ability to multiplex Lower sensitivity due to consumption of analyte during culture stimulation Sample volumes limit assessment of multiple analytes No surface phenotype
Proliferation assay (CFSE)	Direct measure of T cell division Compatible with multi-parametric surface phenotyping Single-cell resolution	Requires fluorescent labeling of lymphocytes prior to antigen stimulation High cell input requirements/limit of detection Limited throughput/scalability
Luminex	Multi-analyte detection/multiplexing High-throughput scalability/batch analysis	No surface phenotype Poor resolution/limit of detection Lower sensitivity due to consumption of analyte during culture stimulation
ICS	Multi-analyte detection/Multiplexing Compatible with multi-parametric surface phenotyping Single-cell resolution Functional phenotyping	Inhibition of secretion required for analyte detection High cell input requirements/limit of detection Limited throughput/scalability Destructive method

experienced memory cell populations occur in the 1:10⁴ to 1:10⁶ frequency range among all B and T cells, and are stably maintained for years, if not decades, even in the absence of antigen re-encounter^{16,17}.

While standard flow cytometric approaches are well-suited for studying antigen-specific T cells that exist in the 1:10³ – 1:10⁴ frequency range, these methods are not ideally suited for detecting memory T cells that exist at lower frequencies¹⁸ (Table 1). Yet memory T cells specific for most antigens occur in substantially lower frequencies. ImmunoSpot® assays represent an alternative approach to these traditional flow cytometry methodologies since they are more sensitive and reliably detect antigen-specific memory T cell populations as rare as 1:10⁶^{17, 19}. The sensitivity of ImmunoSpot® assays is not only essential for the mere identification of antigen-specific memory T cells in peripheral blood, but in particular for segregating these cells into subpopulations to ascribe their distinct effector functions. To this end, immune monitoring aims to establish both the frequency of antigen-specific memory T cells in the blood and their effector profile as a measure of immunity²⁰.

Antigen-specific T cells belong to either the CD4⁺ or CD8⁺ lineage. While CD8⁺ T cells preferentially recognize antigens that are biosynthesized by infected or transformed host cells, CD4⁺ T cells typically target protein antigens acquired from the extracellular space¹. Upon antigen encounter and appropriate co-stimulatory cues, both CD4⁺ and CD8⁺ T cells differentiate into discrete memory cell lineages that secrete distinct and often mutually exclusive sets of cytokines^{15, 21}. Several distinct CD4⁺ T helper (Th) subsets have been identified, and in particular the Th1, Th2 and Th17 lineages have been most extensively characterized. However, additional CD4⁺ T cells subsets have gained notoriety in recent years and include the Th9 and Th22 subsets that secrete IL-9 and IL-22 respectively^{22, 23}. Moreover, follicular T cells (Tfh) migrate into B cell follicles due to upregulation of CXCR5 and support germinal center reactions and antibody production through production of IL-21²⁴. Furthermore, FoxP3⁺ regulatory T cells (Treg) are a specialized CD4⁺ subset that exhibit immunosuppressive activity²⁵.

In the context of the well-characterized Th1, Th2 and Th17 lineages, Th1 cells are capable of interferon

gamma (IFN- γ), but not interleukin 4 (IL-4) or interleukin 17 (IL-17) secretion^{26, 27}. Th1 cells are also important for promoting an inflammatory response that confers protection against most viruses²⁸. Th2 cells secrete IL-4, but not IFN- γ or IL-17, and likely originally evolved to convey anti-parasite immunity²⁹. However, especially in industrialized countries, Th2 cells are more commonly associated with allergies³⁰. Lastly, Th17 cells secrete IL-17, but not IFN- γ or IL-4, and are critical for mounting effective immune responses against intracellular pathogens and fungi³¹. Autoantigen-reactive Th17 cells are also thought to be the main drivers of autoimmune pathology³².

Whereas secretion of IFN- γ , IL-4 and IL-17 are mutually exclusive to the Th1/Th2/Th17 lineages, respectively, production of IL-9, IL-21 or IL-22 is not necessarily restricted to a single T helper subset. Specifically, IL-9 can be produced by several subsets of CD4⁺ T cells in addition to Th9 cells, including the Th2, Th17 and Treg lineages³³. Moreover, Th9 polarized cells can also produce IL-10 and IL-21²³. Th9 cells have been associated with immunity against parasites and tumors, but are also linked to allergic inflammation and autoimmune diseases³⁴. Likewise, IL-21 is produced by several CD4⁺ T cell subsets, including Th9 and Th17, and therefore is not restricted to the Tfh subset³⁵. Additionally, while expression of IFN- γ , IL-17 or IL-5 is rare amongst human Tfh cells³⁶, these cells are also defined by IL-4 production³⁷. Furthermore, Tfh cells in mouse models can acquire expression of IFN- γ or IL-17 under certain circumstances^{38, 39}. Tfh are closely associated with protective humoral immunity through their ability to promote immunoglobulin class-switching and affinity maturation in B cells. However, increased frequencies of circulating Tfh-like cells also occur in certain autoimmune diseases associated with elevated levels of autoantibody⁴⁰⁻⁴³. Lastly, the IL-22 cytokine is produced by both Th22 and Th17 lineages^{44, 45}. Th22 cells are mostly associated with immune responses and tissue regeneration occurring in the skin⁴⁶. However, Th22 cells and/or IL-22 levels are also negatively associated with Crohn's diseases⁴⁷ and cancer^{48, 49}.

Due to the fact that the Th1/Th2/Th17 memory T cell lineages elicit fundamentally different inflammatory response patterns upon antigen re-encounter, it is insufficient for immune diagnostic purposes to enumerate only the frequency of antigen-specific T cells. Instead, it is essential to understand whether the Th1, Th2 or Th17 cell lineage is dominant within the antigen-specific memory T cell pool. By counting the number of individual antigen-specific T cells in PBMC that secrete IFN- γ vs. IL-4 vs. IL-17 following cognate antigen stimulation, immune monitoring seeks to establish the ratio of Th1/Th2/Th17 memory T cells in the blood, and thus to characterize the quality of T cell immunity in individual test subjects.

An important factor that needs to be considered when

establishing the ratio of Th1/Th2/Th17 antigen-specific T cells in the blood is that the kinetics of IFN- γ , IL-4 and IL-17 production are very different. IFN- γ production by Th1 cells peaks 24h after antigen encounter, IL-4 secretion by Th2 cells peaks at 48h, and IL-17 production by Th17 cells peaks 3-4 days after antigen stimulation (not even being detectable at 24h/48h)⁵⁰. As a consequence of these non-overlapping secretion kinetics, Th2 and Th17 antigen-specific T cells are likely to go undetected when immune monitoring is performed at a single time-point, typically within 6-24h following antigen stimulation, as has traditionally been done.

Immune monitoring also aims to establish the life history of memory T cells. Antigen-specific memory T cells' ability to contribute to host defense is particularly high if these T cells are still in their prime and capable of proliferative expansions⁵¹. In contrast, as a consequence of chronic antigen exposure, T cells can be driven to exhaustion and become dysfunctional⁵². The cytokine secretion profile observed following antigen stimulation therefore enables distinction between these two extremes, as well as intermediate stages of a T cell's life history (Table 2)⁵¹.

In the remainder of this mini-review we will focus on the secretory signature of CD8⁺ memory T cell subpopulations. While the list of CD8⁺ T cell subsets continues to expand, and is beyond the scope of this review, the underlying theme remains the same. Namely, naïve CD8⁺ T cells (T_N) lack cytolytic activity and fail to produce cytokines following their initial antigen encounter⁵³. However, within 5 days of antigen receptor engagement, naïve CD8⁺ T cells acquire effector functions and produce high levels of perforin and granzyme B (GzB) enabling them to efficiently kill infected or transformed target cells⁵⁴. These T cells are now termed terminal effector cells (T_{TE}). They are capable of producing large quantities of GzB, perforin, IFN- γ and TNF- α , but T_{TE} produce little if any IL-2^{55, 56}. In the absence of ongoing

Table 2. Dissection of CD8⁺ memory T cell subpopulations based on analyte secretion profiles. At different stages of their differentiation, the specified CD8⁺ memory T cell subpopulations secrete distinct patterns of interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), interleukin 2 (IL-2) and granzyme B (GzB). The subpopulations are, as specified in the text: naïve T cells, T_N ; stem cell-like memory T cells (T_{SCM}); central memory T cells (T_{CM}); polyfunctional memory T cells (T_P); Terminal effector T cells (T_{TE}); silent killer effector cells (T_{SK}); dysfunctional T cells (T_D); and senescent T cells (T_{SC}).

CD8 ⁺ T cell Subset	Proliferation	IFN- γ	TNF- α	IL-2	GzB
T_N	+++	-	-	-	-
T_{SCM}	+++	-	-	+++	-
T_{CM}	++	+++	+/-	+/-	-
T_P	+	+++	+++	++	-
T_{TE}	-	+++	+++	-	+++
T_{SK}	-	-	-	-	+++
T_D	-	+/-	+/-	-	-
T_{SC}	-	-	-	-	-

antigen receptor signaling, activated effector CD8⁺ T cells return to a quiescent state to become central memory cells (T_{CM})⁵⁷. T_{CM} are capable of IFN- γ secretion within 24h of antigen recall but have (temporarily) lost the ability to secrete GzB and perforin. CD8⁺ T_{CM} cells also secrete little to no TNF- α or IL-2 within the first 24h after antigen re-encounter, but within 3-4 days of antigen stimulation these cells can convert again into T_{TE}⁵⁶.

Another CD8⁺ memory T cell subset termed “stem cell-like memory” (T_{SCM}) has gained increasing attention in recent years⁵⁸. In the context of adoptive cancer therapy, it was observed that transfer of fully differentiated CD8⁺ T cell effectors resulted in less tumor clearance and worse clinical outcome relative to injection of less-differentiated CD8⁺ T cells^{59, 60}. T_{SCM} are capable of extensive proliferation following antigen re-encounter, a trait that is progressively lost by other CD8⁺ memory cell subpopulations following repetitive antigen stimulation. Due to their self-renewing potential and longevity, T_{SCM} can give rise to large numbers of T_{TE} cells following antigen re-encounter, and are therefore especially important when a sustained CD8⁺ T cell response is required. Following antigen stimulation, T_{SCM} secrete negligible amounts of IFN- γ and TNF- α , and lack expression of GzB. However, T_{SCM} produce abundant quantities of IL-2⁵⁶.

There is also a subpopulation of terminal effector CD8⁺ T cells, termed “silent CD8⁺ killer cells” (T_{SK}) that do not secrete IFN- γ , and instead exert their cytolytic effector function through the production of GzB and perforin, without eliciting a local inflammatory response⁶¹.

Amongst CD8⁺ memory T cells there is a subpopulation that co-expresses IFN- γ with IL-2 and TNF- α . These so-called “polyfunctional” T cells (T_p) represent a minor percentage of the total antigen-specific CD8⁺ T cell pool, but their presence in elevated frequencies has been linked to protective immune responses against infectious diseases and cancer⁶².

CD8⁺ T cells will undergo exhaustion if repetitively stimulated in the context of a high antigen burden, as frequently occurs in the context of anti-tumor immunity, autoimmunity and chronic infections. “Dysfunctional” CD8⁺ T cells arise (T_d) that have lost their proliferative capacity and exhibit impaired cytolytic activity and reduced cytokine secretion⁶². While not completely lacking functionality, these cells possess attenuated effector functions to avoid excessive collateral damage in the tissue(s) in which the antigen persists. With ongoing stimulation, these CD8⁺ T cells will eventually burn out and become “senescent” (T_{sc}). Senescent CD8⁺ T cells fail to secrete the pro-inflammatory cytokines IFN- γ , TNF- α or IL-2 in response to antigen stimulation. Instead, T_{sc} secrete IL-6 and IL-8, which primarily serves to promote tissue repair but also promotes tumor growth⁶³.

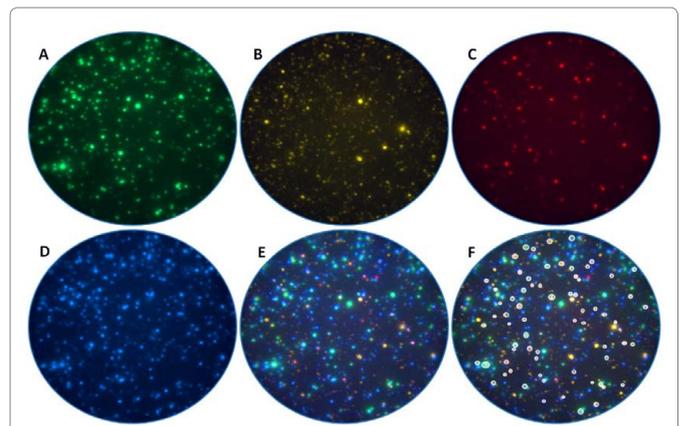


Figure 2: Dissection of CD8⁺ T cell subpopulations using a four-color ImmunoSpot[®] assay

Human peripheral blood mononuclear cells were stimulated with antigen and the elicited interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), interleukin 2 (IL-2) and/or granzyme B (GzB) captured on the membrane around the individual T cells that secreted the respective analyte(s). The individual analytes are selectively detected in the (A) IFN- γ , (B) TNF- α , (C) IL-2 and (D) GzB channels, respectively. Note the lack of signal cross-bleeding, which permits identification of each analyte in its own color plane without the need for compensation. (E) Composite overlay of the four individual channels, enable identification of analyte co-expression patterns by individual CD8⁺ cells. (F) Polyfunctional CD8⁺ T cells (T_p) that secrete IFN- γ , TNF- α , IL-2 and GzB are highlighted using white outlining.

As shown in Table 2, many of the CD8⁺ T cell subpopulations described above can be identified/segregated based on their secretory signature through measurement of IFN- γ , TNF- α , IL-2 and GzB production following antigen challenge. Since these subpopulations occur in rather low frequencies, four-color ImmunoSpot[®] assays particularly lend themselves to dissecting the expression/co-expression pattern of these analytes at single-cell resolution⁶⁴. For the accurate identification of these distinct CD8⁺ subpopulations, it is critical that each analyte is measured without signal overlap. This is accomplished by using combinations of fluorochromes that can be completely separated, and each color plane analyzed, without the need for compensation, essentially as a single-color ELISPOT assay. Next, images containing the “spots” for the different color planes are superimposed to identify cells that co-express analytes (see Figure 2). This cannot be accomplished by simply matching centers of masses for the individual spots because analyte production is asynchronous and T cells migrate during the assay’s capture phase which lasts 24h or more. Thus, tolerances need to be accommodated as we have described previously⁶⁵. Moreover, it has been shown that in the low frequency range in which antigen-specific T cells occur, the chance for random overlays of single-positive spots occurring is essentially nonexistent. Once single- vs. multiple-analyte-producing cells are identified, which is done automatically

by the ImmunoSpot® software, the data is immediately converted into flow cytometry standard (FCS) format. Using standard software, the various analyte-producing populations can be analyzed for co-expression levels and patterns in a very similar fashion as is traditionally performed following intracellular cytokine staining using a flow cytometer.

In conclusion, multi-color ImmunoSpot® assays are ideally suited for assessing rare populations of antigen-experienced T cells and enable segregation of functionally distinct T cell lineages on the basis of their secretory signatures. Additionally, taking into account the assay's ease of implementation, efficient utilization of white blood cells, high-throughput capability, automated evaluation, as well as audit trails generated for regulatory compliance, multi-color ImmunoSpot® assays have great potential to revolutionize the immune monitoring field through providing detailed information on the magnitude, quality, and antigen life history of T cells.

Conflict of Interest Statement

PVL is the founder and CEO of Cellular Technology Limited (CTL), a company that specializes in ELISPOT testing, producing high-throughput-suitable readers, test kits, and GLP-compliant contract research. GAK, JH and DRR are employees of CTL

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