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Review Article (Invited)

Unraveling T-cell dynamics using fluorescent timer: Insights from the Tocky system

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Understanding the temporal dynamics of T-cell activities is crucial for insights into immune cell function and development. In this study, we show the features of the Timer-of-Cell-Kinetics-and-Activity (Tocky) system, which enables analysis of temporal dynamics of cell activities and differentiation, leveraging Fluorescent Timer protein, which spontaneously changes its emission spectrum from blue to red fluorescence in known kinetics, as reporters. The current study examines the properties of the Tocky system, highlighting the Timer-Angle approach, which is a core algorithm of Tocky analysis and converts Timer Blue and Red fluorescence into Timer Angle and Intensity by trigonometric transformation. Importantly, Tocky analyzes time-related events within individual cells by the two phases of measurements, distinguishing between (1) the temporal sequence of cellular activities and differentiation within the time domain, and (2) the transcription frequency within the frequency domain. The transition from time measurement to frequency analysis, particularly at the Persistent locus that bridges these domains, highlights that system's unique property in what is measured and analyzed by Tocky. Intriguingly, the sustained transcriptional activities observed in cells at the Persistent locus may have unique biological features as demonstrated in activated regulatory T-cells (Treg) and pathogenic T-cells, respectively, using Foxp3-Tocky and Nr4a3-Tocky models. In conclusion, the Tocky system can provide crucial data for advancing our understanding of T-cell dynamics and function.

Key words: Fluorescent Timer protein, Nr4a3, Foxp3, T-cells, Biological Measurement Singularity

Significance

This study presents the Tocky system, a transformative approach for real-time analysis of T-cell transcriptional dynamics through Fluorescent Timer proteins. By identifying Nr4a3 as a key gene linked to TCR signaling, we establish the Nr4a3-Tocky model. The current study highlights the unique property of the Tocky system, identifying the persistent transcriptional dynamics of Tocky as a '*Biological Measurement Singularity*' at the interphase of the time and frequency domains within the Tocky system. The study unveils the unique T-cell dynamics that are associated with the persistent transcription in pathogenic T-cells using Nr4a3-Tocky and activated regulatory T-cells in Foxp3-Tocky.

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Introduction

T-cells as a Dynamic System

The analysis of temporal changes of cellular activities and differentiation in vivo is key to understand how a tissue or a cell population is formed through successive biological steps [1]. This dynamic perspective is important in immunology, especially for investigating T-cells. T-cells, defined as the lymphocytes with a unique variable antigen receptor (T-cell receptor, TCR) possess the distinct ability to recognize antigens specifically. These antigens are typically short peptides presented on a specialized molecule known as the Major Histocompatibility Complex (MHC) [2]. Upon antigen recognition, T-cells initiate proliferation and differentiation, leading to functional responses, such as the cytotoxic activity of $CD8^+$ T-cells and the activation and regulation of other immune cells by $CD4^+$ T-cells.

The thymus plays a pivotal role in the generation of the diverse T-cell receptor (TCR) repertoire, essential for T-cell development and selection. Thymocytes transition from immature Double Negative (DN) and Double Positive (DP) stages to mature Single Positive (SP) cells. During these developmental phases, T-cells undergo TCR gene recombination, which incorporates both predetermined (germline-encoded) and stochastic (randomly generated) elements. This recombination diversifies the antigen-recognizing regions of the TCR and is followed by stringent selection processes. Positive selection ensures T-cells can recognize self-MHC molecules, while negative selection eliminates those with strong self-reactivity, guided by TCR signaling strength [3]. In addition, some thymic T-cells undergoing negative selection may be rescued by expressing the transcription factor Foxp3, specific to regulatory T-cells (Treg, see below) [4].

These selection processes in the thymus and the dynamic developmental events underscore the need for sophisticated measurement tools to analyze T cell responses following antigen recognition [5]. Specifically, *the Timer-of-cell-kinetics-and-activity* (Tocky) system provides crucial data for examining the temporal dynamics of T-cells following TCR signaling [6].

Investigating Foxp3 to Understand $CD4^+$ T-cell-mediated Regulation

The dynamic regulation of T-cells is critically important in the periphery as well. Some $CD4^+$ T-cells may mature into naïve T-cells, emigrate the thymus, and are then maintained in lymphoid tissues, while some other $CD4^+$ T-cells may express Foxp3 in the thymus and are identified as Treg in the periphery.

Treg are a subset of $CD4^+$ T-cells that express Foxp3 and can suppress the activities of other T-cells [7]. Conceptually, Treg are defined by their suppressive activity: upon recognizing an antigen, they do not induce immune response but suppress it [8]. From the TCR perspectives, the majority of Treg are considered to have self-reactive TCRs, recognizing antigens in self-tissues, especially in young animals and humans [9]. This affinity for self-antigens is considered to enable Tregs to suppress unwanted immune responses to self-tissues [10].

However, the concept of Treg harbors two fundamental problems. Firstly, their suppressive activity is measurable only through bulk cell analysis, with no method available for analyzing this activity in individual Treg cells in vivo. The most common approach, the in vitro Treg suppression assay, is a mixed cell culture experiment involving Treg and 'responder' T-cells; it assesses the suppression of the proliferation of the responder T-cells in the presence of Treg [11], which may not reflect in vivo T-cell regulation. The alternative approach includes the adoptive transfer of Treg into recipient mice in which a certain type of inflammation is induced [12], but it is not possible to identify which Treg cells are in action. Secondly, the notion of Treg as a stable and distinct lineage is considerably supported by the 'unique dynamics' of neonatal Treg differentiation reported by Asano et al. [13] – which was claimed to reveal the cause of neonatal thymectomy-induced autoimmunity. However, this notion has been compromised due to reproducibility issues in the Asano et al. study, as previously demonstrated [14]. These two problems have led to significant confusions in the research field, which is an ongoing issue. Given the considerable plasticity of Treg and the dynamic regulation of Foxp3 expression [15,16], it is essential to further investigate Treg as part of the $CD4^+$ T-cell system, focusing on Foxp3 expression and TCR signaling dynamics [4,14].

Despite the challenges in Treg studies, evidence on Foxp3 is substantial. Foxp3 expression is experimentally measurable and crucially delineates Treg differentiation and function. This will be elaborated with Foxp3-Tocky as below. Furthermore, the analysis of TCR sequences is feasible by recent advancements in sequencing technologies. Advances in sequencing enable TCR sequence analysis, with the Nr4a3-Tocky system offering solutions to immunological issues, as discussed later.

Investigating Temporal Dynamics of Cellular Activities In Vivo Using Fluorescent Timer Protein

To investigate the temporal dynamics of T-cell differentiation and activity, I considered using fluorescent timer proteins in 2013 due to their ability to irreversibly change emission spectra over time, which should allow analysis of temporal dynamics of Foxp3 and TCR signaling as warranted by the historical considerations above. Preliminary experiments demonstrated that Fluorescent Timer (hereafter Timer) proteins, which change its emission spectrum spontaneously and

irreversibly, can provide a promising approach for the purpose, if used as a reporter for key genes or proteins. The applications of Timer proteins include developmental cell biology studies using Zebrafish [17] and Caenorhabditis elegans [18], functional characterization of pancreatic islet cells [19], and cell cycle [20]. In immunology research, fluorescent timer proteins are becoming essential for studying immune cell dynamics, which is typically analyzed using flow cytometry [6,21–26].

However, to effectively analyze flow cytometric Timer data, particularly in immunology and other areas of cell biology, data-oriented and quantitative methods are essential. To address this, the Tocky approach aims to experimentally analyze the temporal dynamics of transcription using Timer as a reporter gene [6]. This approach decodes the temporal information encoded in Timer fluorescence in individual cells. We selected *fastFT* [6], which was developed by Subach et al. (2009), a mutant derivative of mCherry [27]. Immediately after translation, mCherry features a blue-type chromophore that initially emits blue fluorescence. This spontaneously shifts to red through a self-catalytic activity during protein maturation. The transformation occurs as the protein translates and folds, involving the removal of a proton from the chromophore, thereby altering its emission spectrum from blue to red. Engineered to mature more slowly than mCherry, the fastFT variant facilitates a delayed transition, enabling more precise temporal tracking of protein synthesis and maturation [27]. Our experimental measurements determined the half-life of the blue and red fluorescence, approximately 4 hours and 120 hours, respectively [21]. This dichotomy underscores the ability of blue fluorescence to capture real-time transcriptional activities, while red fluorescence indicates transcriptional history [6].

Development of the First Tocky Mouse Model

In 2013, upon establishing my new laboratory with support from the Biotechnology and Biological Sciences Research Council (BBSRC), I aimed to innovate an experimental system capable of dissecting the temporal dynamics of T-cell activities post-TCR activation. Recognizing the limitations of existing models, such as the Nur77 GFP reporter mouse, which lacks temporal resolution [3], I conceived the use of a Fluorescent Timer reporter transgenic approach to address my question. This approach would involve targeting a gene with a direct correlation to TCR signaling, providing a more accurate temporal analysis [6]. I employed Canonical Correspondence Analysis (CCA), a multidimensional analysis method that I introduced and adapted for cross-analyzing transcriptome datasets [28], to correlate gene expression with cellular phenotype and differentiation states in differentiating T-cells [29,30]. This analysis pinpointed *Nr4a3* as the gene with the strongest correlation to TCR signaling in both thymic and peripheral T-cells [6]. Although Nr4a1 (Nur77) and the NF-kB gene Rel were also correlated with TCR signaling, their associations were not as pronounced as that of Nr4a3. Prior studies have utilized the Nur77 GFP reporter mouse strain to measure TCR signal ‘strengths’ [3]; however, GFP's stability severely limits its temporal resolution. Moreover, GFP expression in the Nur77-GFP model occurs in T-cells which have not received cognate antigen signalling, compromising its utility for analyzing antigen-reactive T-cell activities. Consequently, I embarked on an ambitious project to innovate a completely novel approach to dissect temporal dynamics of cell activities. Thus, I developed the novel Fluorescent Timer reporter mouse strain for the Nr4a3 gene, in which Timer expression is synchronized with Nr4a3 transcription following TCR signaling. I designated this approach as the Nr4a3-Tocky model [6]. Using Nr4a3-Tocky, cognate antigen signaling is effectively captured by Timer expression and allows analysis of T-cell dynamics post TCR signaling (Figure 1).

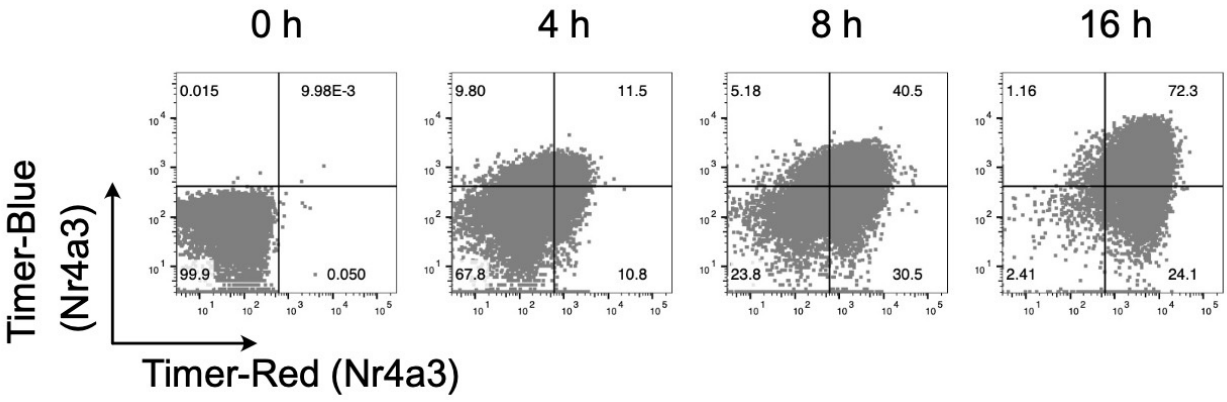

Figure 1 Fluorescent Timer expression following TCR signaling in Nr4a3-Tocky T cells. $CD4^+$ T-cells from Nr4a3-Tocky mice were stimulated by antigen and analyzed at the indicated time points by flow cytometry. The expression of Timer-Blue indicates new and active transcription while Timer-Red shows historical accumulation of Timer protein. Data modified from [6].

The Development of Tocky as a Tool to Investigate T-Cell and B-Cell Dynamics

The Tocky system utilizes flow cytometry as a key method for the quantitative analysis of temporal transcription dynamics [6]. Flow cytometry measures the fluorescence emitted by individual cells, allowing for the precise detection of the color changes of the fastFT protein. This methodology enables the differentiation between cells exhibiting blue fluorescence (indicative of newly synthesized Timer proteins) and those emitting red fluorescence (representing matured proteins). A trigonometric transformation allows the conversion of flow cytometric Timer data into Timer Angle and Timer Intensity (Figure 2A) (6). This angle, determined from the degree of blue versus red fluorescence, provides a quantitative measure of transcriptional activity at different stages. The trigonometric transformation allows succinct data presentation and quantitative analysis of the Tocky data (Figure 2B). Nr4a3 Tocky has been established as a method to analyze B-cell and T-cell dynamics [6].

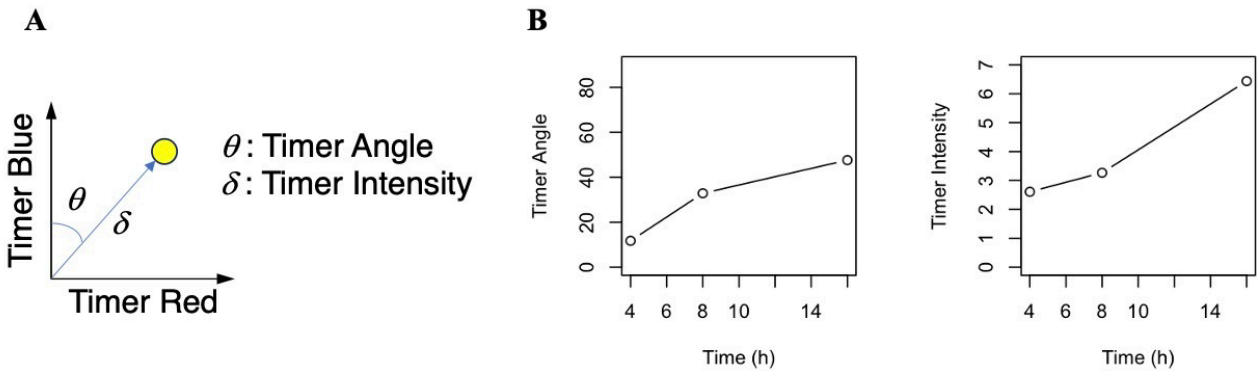

Figure 2 Definition of Timer Angle and Timer Intensity. (A) The figure illustrates the conceptual representation of the Timer Angle (θ) and Timer Intensity (δ) in the context of the Tocky system. The Timer Angle is determined by the arc tangent of the ratio of fluorescence intensities from Timer Blue to Timer Red emissions, representing the stage of transcriptional activity within a cell. The Timer Intensity is a radial measure of the overall fluorescence magnitude, reflecting the total amount of Timer protein present. The depicted yellow circle represents an individual cell's position in the Blue-Red plane, with its corresponding Timer Angle and Timer Intensity value. (B) The mean values of Timer Angle (left) and Timer Intensity (right) of the data in Figure 1 are shown. The progression of Timer Angle and Timer Intensity is effectively visualized.

Timer Angle: Quantifying Transcription Dynamics

I further developed innovative approaches to analyze Timer expression data. Notably, the Timer Angle approach enable the quantitative analysis of transcriptional dynamics and temporal progression in individual cells [6]. Cells with a Timer Angle close to 0 degrees, characterized by predominantly blue fluorescence, indicate recent activation of transcription. This is because the fastFT protein, immediately after translation, emits blue fluorescence before it matures into a red-emitting form with the half-life 4 hours [21]. As the Timer protein matures, transitioning from blue to red fluorescence, the Timer Angle in the cells increases. When the Timer Angle reaches approximately 45 degrees, it signifies a balanced state where the rate of new blue-emitting protein production is equal to the rate of maturation to red-emitting forms, representing a steady state of transcription. Cells that exhibit a Timer Angle progressing towards 90 degrees indicate a predominance of matured red protein, suggesting either a slowdown or cessation of new Timer protein production. This progression of the Timer Angle from 0 to 90 degrees provides a nuanced view of transcriptional dynamics, capturing both the initiation and the persistence of transcriptional activity over time.

In the Tocky system, the progression of the Timer Angle is intrinsically linked to the temporal dynamics of Timer transcription, providing key insights into cellular transcriptional behavior. When Timer transcription is sustained and continuously active, the progression of the Timer Angle is slower [6]. This is because new Timer proteins are constantly synthesized, maintaining a significant level of blue fluorescence, even as some proteins mature and emit red fluorescence. As a result, the balance between blue and red fluorescence shifts gradually, leading to a slower increase in the Timer Angle. In contrast, when Timer transcription is short and transient, there is an initial burst of blue fluorescence that quickly transitions to red as the proteins mature, without replenishment from new protein synthesis. This results in a more rapid progression of the Timer Angle towards higher values, indicating a quicker transition from predominantly blue to predominantly red fluorescence [6]. This dependency of Timer Angle progression on the transcriptional activity of the Timer protein allows the Tocky system to distinguish between cells with different transcriptional dynamics, thereby providing a nuanced understanding of the underlying cellular processes.

Composite Measurement of Time and Frequency by Timer Angle: Biological Measurement Singularity

The Timer Angle in the Tocky system is crucial for understanding transcription dynamics, distinctly represented in two phases: 0–45 degrees and 45–90 degrees [6]. The 0–45 degree range is indicative of the time elapsed since the initiation of transcription. This phase focuses on the early stages of transcription, where newly synthesized fastFT proteins emit blue fluorescence. As the Timer protein transitions towards a mature red-emitting state, the Timer Angle increases, approaching 45 degrees, which marks a balanced state of continuous transcription. Conversely, the 45–90 degree range reflects the frequency of transcription. In this phase, the accumulation of red fluorescence signifies a shift from active transcription towards a state where the maturation of the Timer protein dominates. This higher angle indicates either a reduced rate of transcription, and consequently that of new protein synthesis, or the predominance of protein maturation. Therefore, the Timer Angle offers a unique composite measurement of time in the Tocky system: it captures timing and temporal order in the 0–45 degree range (*time domain*) and transcription frequency in the 45–90 degree range (*frequency domain*) [6].

In contrast to the theoretical consideration above, flow cytometry data includes inherent variations and electric noise in any fluorescence data, which typically follows Gaussian distribution. To address these challenges, the Tocky Locus approach has been developed for data segmentation (Figure 3). This method divides the Timer Angle range of 0˚–90˚ degrees into five distinct loci: New [0˚], New-to-Persistent-transitioning (NPt, (0˚–30˚]), Persistent [30˚–60˚), Persistent-to-Arrested-transitioning (PAt, [60˚–90˚)), and Arrested [90˚]. Each locus represents a specific phase in the transcriptional activity of the cell: 'New' indicates recent transcription activation, 'NPt' and 'PAt' mark the transitioning phases, 'Persistent' denotes ongoing transcription, and 'Arrested' signifies a cessation of transcriptional activity. This segmentation not only manages experimental variability and noise but also provides a refined analysis of cellular transcription dynamics. Particularly, the 'Persistent' locus can include both (1) newly transcribing cells that are moved from New and NPt; and (2) cells that have reactivated Timer expression, increasing the frequency of transcription. It is an inherent property of the system that these two types of trajectories may not be immediately distinguished without the combined use of other experimental methods.

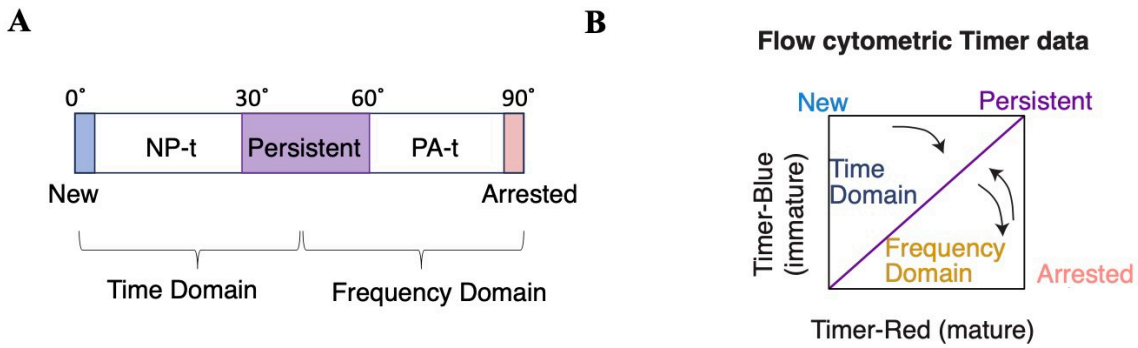

Figure 3 The Tocky Locus Approach and The Composite Measurement of Time and Frequency Domains in the Tocky System. (A) The diagram delineates the *Tocky Locus* approach to segmenting Timer Angles into distinct phases of transcriptional activity. This method divides the Timer Angle range of 0˚–90˚ degrees into five distinct loci: New (0˚), New-to-Persistent-transitioning (NPt, 0˚–30˚), Persistent (30˚–60˚), Persistent-to-Arrested-transitioning (PAt, 60˚–90˚), and Arrested (90˚). Crucially, the Timer Angle of 0–45 degrees corresponds to the Time Domain, capturing the temporal order of cell progression after activating Timer transcription, while the Timer Angle of 45–90 degrees corresponds to the Frequency Domain, indicating the transcription frequency, with 45˚ the most frequent transcription, i.e. Persistent. (B) The time and frequency domains are depicted in a schematic figure of flow cytometric data analyzing Timer expression. Arrows indicate the directions of Timer Angle progression, emphasizing the 'reversible' nature of cell movements in the frequency domain.

Interestingly, the Persistent locus in the Tocky system provides a unique *Biological Measurement Singularity*, mirroring aspects of singularities in mathematics and physics (Figure 3A and 3B). Like physical or mathematical singularities, it represents a critical transition point — in this case, from the time domain (measuring transcription initiation) to the frequency domain (measuring transcription frequency). This similarity is observed in its role as a juncture where conventional measurements transition to a different state, akin to phase transitions or theoretical extremes in physics and mathematics [31,32]. However, uniquely, Biological Measurement Singularity in the Tocky system is based on experimentally measurable transcriptional dynamics. The singularity arises when the analysis of time-dependent processes becomes indistinguishable from frequency analysis, occurring when transcriptional dynamics exceed a certain

threshold of frequency and continuity. Thus, the Biological Measurement Singularity of Tocky stems from the maturation properties of the Fluorescent Timer protein, which enables the analysis of time-dependent process limited by the protein maturation kinetics.

Immunological Significance of T-cells in the Persistent Locus

In addition to the unique property of the Persistent locus as discussed above, recent studies have highlighted the significance of the Persistent locus in the Tocky system. Our previous work utilized Nr4a3-Tocky to examine myelin-specific T-cells in central nervous system (CNS) tissue using a neural inflammation model, Experimental Autoimmune Encephalomyelitis (EAE), which is a murine model of Multiple Sclerosis [6]. In this model, autoimmune neural inflammation can be induced by the immunization of myelin protein (namely, Myelin Oligodendrocyte Glycoprotein, MOG) in mice [33]. In our experiment using Nr4a3-Tocky mice [6], $CD4^+$ T-cells in the draining lymph nodes of the immunization site include less than 1% of MOG-specific T-cells (Figure 4A). Among these T-cells, ~10% of cells were identified in the Persistent Locus (Figure 4B). These mean that only ~0.1% of $CD4^+$ T-cells were MOG-specific and had persistent TCR signals in the lymph nodes from EAE mice. In contrast, the central nervous system in the EAE mice included a higher number of MOG-specific T-cells. Approximately 5% of $CD4^+$ T-cells in inflamed spinal cord in EAE mice were MOG-specific and ~40% of them were found in the Persistent Locus. Thus, ~2% of spinal cord-infiltrating $CD4^+$ T-cells were MOG-specific and persistently recognized MOG antigen. Although the percentages are small, MOG-specific $CD4^+$ T-cells are known as pathogenic T-cells and drive the neuroinflammation in the EAE model [34]. Intriguingly, those 'rare' T-cells demonstrate the unique dynamics as identified in the Persistent locus in Nr4a3-Tocky mice. This suggests that a sustained, high-frequency transcriptional activity indicates a critical phase in the cellular response within the CNS in the EAE model. Thus, the unique dynamics of TCR signaling were found uniquely in the pathogenic T-cells in action only during inflammation.

In a parallel study with Nr4a3-Tocky, we developed Foxp3-Tocky to investigate Treg cell dynamics in vivo [21]. This indeed led to the intriguing observation that Treg cells that persistently transcribe Foxp3 (i.e. in the Persistent locus) show the profile of activated Treg or '*effector Treg*', which highly express immune checkpoint molecules such as CTLA-4 and ICOS and show augmented suppressive activities [21].

The convergence of these studies on the Persistent locus as a unique T-cell status, using the two different Tocky mouse lines for the two different T-cell populations, suggests that the persistent transcriptional activity is associated with a dynamically transitioning and biologically unique status of T-cells. This resonates with Biological Measurement Singularity in the Persistent locus in the Tocky system, where cells exhibit a pivotal state of transcriptional activity, crucial for understanding their functional roles in immune responses and disease processes. Given the analysis method to use flow cytometry, the identification of unique cell population accumulated in the Persistent locus is dependent on the overall percentage of cells in the locus per cell population. However, it is equally intriguing to analyze spatial accumulation of cells, ideally in a large scale. Here the transscale microscopy AMATERAS [35] is expected to provide an effective method to identify any rare population clustered in a unique location in tissues.

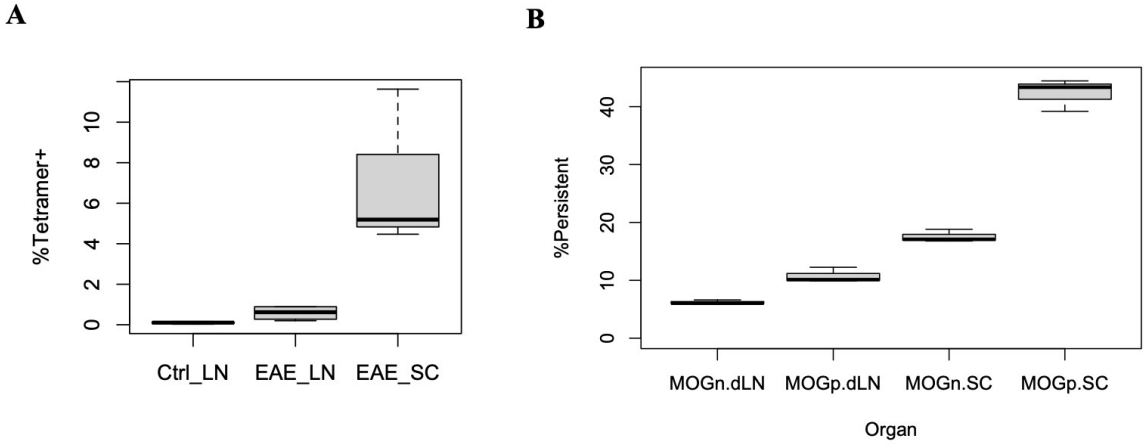

Figure 4 The 'rare' Persistent cells induce neural inflammation. Experimental Autoimmune Encephalomyelitis (EAE) was induced in Nr4a3-Tocky mice. (A) T-cells in draining lymph nodes (LN) and spinal cord (SC) from EAE mice and control (ctrl) mice were subsequently analyzed by flow cytometry using a tetramer specific to Myelin Oligodendrocyte Glycoprotein (MOG). (B) Timer expression was analyzed by the Tocky approach and the percentage of cells in the Persistent locus were analyzed in the $CD4^+$ T-cell fraction of MOG-specific T-cells (MOGp) and non-specific T-cells (MOGn) in draining lymph nodes (dLN) and the spinal cords (SC). The data were originally reported in [6] and re-analyzed for the plots in this figure.

Conclusion

The development of the Tocky system has advanced our capability to analyze the temporal dynamics of T-cells during their differentiation and activities. The Nr4a3-Tocky model successfully captures the temporal dynamics of T-cells following TCR signalling. The Foxp3-Tocky model allows analysis of Treg dynamics through revealing nuanced temporal dynamics of Foxp3 transcription. Importantly, the current study has highlighted the unique property of the Tocky system: it measures two types of time measurements by the time domain, which reveals the sequence of biological events, and the frequency domain, which analyzes transcriptional frequency, with cells with persistent transcriptional activities identified in the interface of the two domains, or the Persistent Tocky locus. Interestingly, T-cells in the Persistent locus show unique activities in the two Tocky models, further supporting the cellular dynamics as Biological Measurement Singularity. In conclusion, the Tocky system allows unveiling temporally dynamic cell differentiation and activities and contributing to our understanding of immune regulation and the development of diseases.

Conflict of Interest

The author declares no conflict of interest.

Author Contribution

MO confirms sole responsibility for all aspects of the manuscript. This includes the conception and design of the study, data collection, analysis and interpretation of results, and manuscript preparation.

Data Availability

The original experimental data in this study were previously reported [6].

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