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# OMIP-079: Cell cycle of $CD4^+$ and $CD8^+$ naïve/memory T cell subsets, and of Treg cells from mouse spleen

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## Abstract

A multicolor flow cytometry panel was designed and optimized to define the following nine mouse T cell subsets: Treg ($CD3^+$ $CD4^+$ $CD8^-$ $FoxP3^+$), $CD4^+$ T naïve ($CD3^+$ $CD4^+$ $CD8^-FoxP3^-$ $CD44^{int/low}$ $CD62L^+$), $CD4^+$ T central memory ($CD3^+$ $CD4^+$ $CD8^-$ $FoxP3^-$ $CD44^{high}$ $CD62L^+$), $CD4^+$ T effector memory ($CD3^+$ $CD4^+$ $CD8^-$ $FoxP3^-$ $CD44^{high}$ $CD62L^-$), $CD4^+$ T EMRA ($CD3^+$ $CD4^+$ $CD8^-$ $FoxP3^-$ $CD44^{int/low}$ $CD62L^-$), $CD8^+$ T naïve ($CD3^+$ $CD8^+$ $CD4^-$ $CD44^{int/low}$ $CD62L^+$), $CD8^+$ T central memory ($CD3^+$ $CD8^+$ $CD4^-$ $CD44^{high}$ $CD62L^+$), $CD8^+$ T effector memory ($CD3^+$ $CD8^+$ $CD4^-$ $CD44^{high}$ $CD62L^-$), and $CD8^+$ T EMRA ($CD3^+$ $CD8^+$ $CD4^-$ $CD44^{int/low}$ $CD62L^-$). In each T cell subset, a dual staining for Ki-67 expression and DNA content was employed to distinguish the following cell cycle phases: $G_0$ ($Ki67^-$, with 2n DNA), $G_1$ ($Ki67^+$, with 2n DNA), and S-$G_2$/M ($Ki67^+$, with 2n < DNA ≤ 4n). This panel was established for the analysis of mouse (C57BL/6J) spleen.

**KEYWORDS**
cell cycle, DNA content, flow cytometry, Ki-67, mouse T cells, spleen

## 1 | BACKGROUND

The periodicity of cell proliferation is a fundamental aspect of biology that, for example, discriminates neural stem cells from terminally differentiated neurons. Likewise, it is a key feature of adaptive immunity that depends upon clonal expansion of primed T and B cells with appropriate antigen specificity, thereupon generating a vast progeny of short-lived effector cells and a few long-lived memory cells. There are two main subsets of T cells, $CD4^+$ and $CD8^+$ T cells, having a predominant helper and cytotoxic effector function, respectively. Maintenance of memory $CD4^+$ and $CD8^+$ T cells over time is a dynamic process, relying on a fine equilibrium among cell death, survival, and low level of homeostatic proliferation [1, 2]. Under steady-state conditions, most T cells in the spleen of untreated mice are quiescent cells, although a tiny proportion divides, possibly reflecting immune responses to unknown environmental antigens and/or cytokine-driven homeostatic proliferation. Such cell cycling in the absence of intentional immunization is more prominent among the so-called memory-phenotype T cells, that share a set of membrane markers with antigen-primed T cells [3]. Furthermore, some Treg cells (a subset of $CD4^+$ T cells with regulatory function defined by the expression of the transcription factor FoxP3 [4]) have an activated/proliferative phenotype, possibly reflecting continuous self-renewal in adult mice [5].

In fact, memory-phenotype T cells comprise a heterogeneous pool of cells of undefined antigen-specificity, that are considered to include T cells primed by environmental antigens, as well as some antigen-inexperienced T cells having self-ligand- and/or cytokine-dependent development [6, 7]. In C57BL/6 mice memory-phenotype T cells have a high expression of CD44, an adhesion molecule that binds to hyaluronic acid, and can thus be distinguished from naïve-phenotype T cells, that have an intermediate/low CD44 expression [6]. Similarly, in humans naïve and memory-phenotype T cells can be identified by high and low expression of CD45RA, respectively [8].

Proliferative potential is one of the features that, together with lymph node (LN) homing capabilities, and effector function, differentiates additional subsets among memory T cells. Thus, central memory (CM) T cells have a LN homing receptor typically expressed by naïve T cells, specifically CD62L, also named L-selectin, a glycan receptor [9], whereas effector memory (EM) T cells lack it [10]. According to this classification, originally proposed for human blood T cells using CCR7, the chemokine receptor for CCL19/CCL21, as a marker [11], T CM are LN-homing cells with high potential to expand after stimulation, while T EM cells are tissue-homing cells able to display rapid effector function [12, 13]. An additional subset, the T EMRA cells, comprises effector memory T cells that re-acquire a naïve phenotype ($CD45RA^+$ in humans, $CD44^{int/low}$ in mice). Based on this, the following four naïve/memory T cell subsets can be identified among mouse $CD4^+$ and $CD8^+$ T cells: $CD44^{int/low}$ $CD62L^+$ naïve, $CD44^{high}$ $CD62L^+$ CM, $CD44^{high}$ $CD62L^-$ EM, and $CD44^{int/low}$ $CD62L^-$ EMRA [10, 14].

While identification of memory T cells with high proliferative potential can impact the success of adoptive transfers [15], accurate measurement of in vivo proliferation is essential to track the dynamics of T cell responses [16]. Proliferation of mouse T cells has been measured by a few cytofluorimetric methods, that can be divided into "static"—that is, those which provide a snapshot of the cell cycle phases at the time of analysis—and "dynamic," that is, those which give information on the proliferation that occurred over a few hours or days prior to the analysis. The most widely used "static" method relies on the Ki-67 marker, an intranuclear protein that supports chromosome architecture organization, and nucleus and nucleolar assembly after cell division [17, 18]. However, Ki-67 is expressed by cells in any phase of cell cycle (i.e., in $G_1$, S, $G_2$, M), while it is only low or absent in quiescent cells (i.e., in $G_0$ state). The "dynamic" methods include carboxyfluorescein succinimidyl ester (CFSE) and Bromodeoxyuridine (BrdU) labelling, which identify proliferating cells that have undergone cell division and S-phase, respectively [2]. We chose to combine Ki-67 staining and DNA content analysis, thereby to distinguish between cells in $G_1$ and those in S-$G_2$/M phases of cell cycle, a discrimination that is relevant to the proliferative fate of the cell. Indeed, cells in S are duplicating their DNA and are committed to proceed into $G_2$/M and divide. In contrast, cells that are in $G_1$ might proceed into S-$G_2$/M, return to $G_0$, or stay in $G_1$ for a prolonged period. Thus, $Ki\text{-}67^+$ cells might not be actively proliferating if they are in $G_1$ phase or are returning to $G_0$. For example, this might be the case for antigen-specific T cell progeny at the end of clonal expansion [19].

In this OMIP, we offer a staining panel for ex vivo cell cycle analysis of $CD4^+$ and $CD8^+$ naïve/memory-phenotype T cell subsets, and of Treg cells from mouse spleen, using Ki-67/DNA dual staining to distinguish cells in $G_0$, $G_1$, and S-$G_2$/M (Table 1; Figure 1). Panel optimization and protocol details are reported in the online Supporting Information. We used standard markers for T cell subset identification. CD3 expression was used to identify T cells, and the mutually exclusive expression of CD4 and CD8 to distinguish $CD4^+$ and $CD8^+$ T cells, respectively. Treg cells were identified among $CD4^+$ T cells based on their expression of the intranuclear protein FoxP3, and by this marker distinguished from conventional $CD4^+$ T cells, that is, $FoxP3^-$ $CD3^+$ $CD4^+$ $CD8^-$ cells. $CD4^+$ and $CD8^+$ naïve/memory T cell subsets were subsequently identified among conventional $CD4^+$ and $CD8^+$ T cells, respectively. The four classical naïve/memory subsets were defined based on their CD62L and CD44 membrane phenotype (see above). For each T cell subset, cells in $G_0$, $G_1$, and S-$G_2$/M were discriminated based on Ki-67 and DNA staining (Table 2; Figure 1).

This OMIP can be exploited for in depth-analysis of T cell cycle in conditions characterized by altered proportions, numbers and

**TABLE 1** Summary table for the application of OMIP-079

| | |
|---|---|
| Purpose | Cell cycle analysis of CD4 and CD8 naïve/memory T cell subsets, and of Treg cells |
| Species | Mouse |
| Cell types | Splenocytes |
| Cross-references | No similar OMIPs |

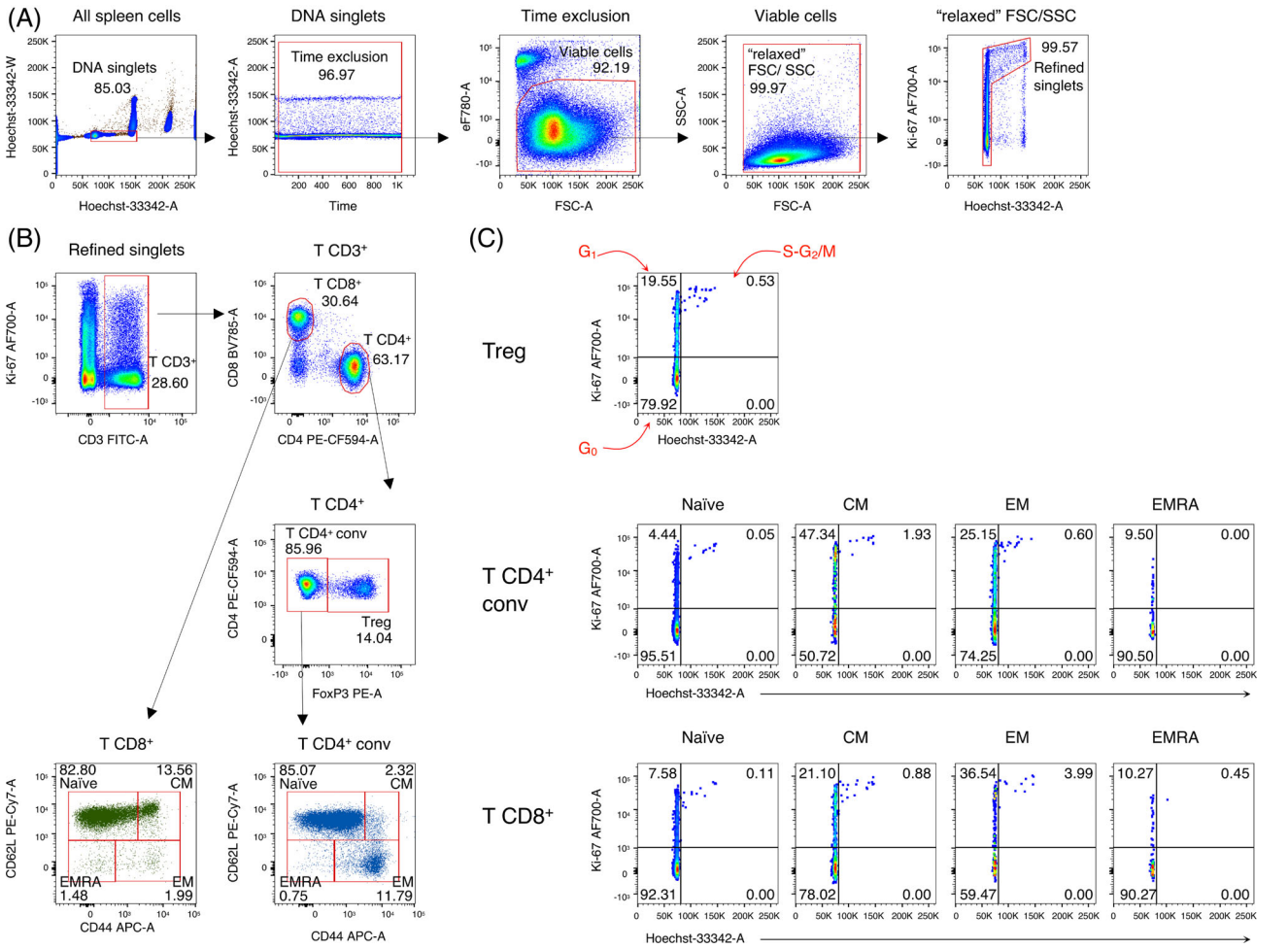


**FIGURE 1** Cell cycle analysis of mouse T cell subsets. Example of analysis of spleen cells from a 3-months old C57BL/6J mouse, using manual gating strategy. (A) Refined gating of viable single cells from the spleen in five steps: (1) DNA singlets. Single cells having 2n ≤ DNA content ≤4n were selected on the Hoechst-33342 area (A) versus (vs) Hoechst-33342 width (W) plot; (2) time exclusion. Stable acquisition over time (seconds) was monitored on the time vs Hoechst-33342-A plot and any events collected in case of pressure fluctuations were excluded; (3) viable cells. Live cells were selected using FSC-A vs eFluor 780 (eF780) viability dye; (4) FSC/SSC "relaxed" gate. A "relaxed" gate was used on the FSC-A vs SSC-A plot, to include highly activated and cycling lymphocytes [19]; (5) refined singlets. A few remaining doublets composed by one cell sitting on top of another (so called "shadow" doublets) were excluded as Ki-67$^{int/-}$ events having >2n DNA content [20]. This gating strategy was used as a base for the subsequent gates. (B) CD3$^+$ T cells were gated on CD3-A vs Ki-67-A plot, then CD4$^+$ and CD8$^+$ T cells on CD4-A vs CD8-A plot. CD4$^+$ Treg cells were distinguished based on their FoxP3 expression from conventional FoxP3$^-$ CD4$^+$ T cells. Subsequently, the following naïve/memory subsets of conventional CD4$^+$ T cells were identified: CD44$^{int/low}$CD62L$^+$ naïve, CD44$^{high}$CD62L$^+$ central memory (CM), CD44$^{high}$CD62L$^-$ effector memory (EM), and CD44$^{int/low}$CD62L$^-$ EMRA. Similarly, naïve/memory subsets were identified among CD8$^+$ T cells. (C) Cell cycle phases of Treg cells and of naïve/memory CD4$^+$ and CD8$^+$ T cell subsets were defined on Hoechst-33342-A vs Ki67-A plot as follows: Cells in $G_0$ were identified as DNA 2n/ Ki67$^-$ (bottom left quadrant); cells in $G_1$ as DNA 2n/ Ki67$^+$ (upper left quadrant); cells in S-$G_2$/M as DNA > 2n/ Ki67$^+$ (top right quadrant) [Color figure can be viewed at wileyonlinelibrary.com]

**TABLE 2** Reagents used for OMIP-079

| Fluorochrome | Specificity | Clone | Function |
|---|---|---|---|
| FITC | CD3 | 145-2C11 | Pan T cell marker |
| APC | CD44 | IM7 | Naïve/Memory subset identification |
| Alexa Fluor 700 | Ki-67 | SolA15 | Quiescence/cell cycle |
| eFluor 780 | Dead cells | N/A | Live/Dead cell discrimination |
| Hoechst 33342 | DNA | N/A | DNA content/cell cycle |
| PE | FoxP3 | FJK-16s | Treg identification |
| PE-CF594 | CD4 | RM4-5 | Helper T cell identification |
| PE-Cy7 | CD62L | MEL-14 | Naïve/Memory subset identification |
| BV785 | CD8 | 53-6.7 | Cytotoxic T cell identification |

Abbreviation: N/A, not applicable.

proliferative state of spleen T cell subsets, for example in aged mice having higher percentages of memory $CD4^+$ and $CD8^+$ T cells, with or without oligoclonal expansion [21–23]; in lymphopenic mice having compensatory T cell proliferation [24]; or in genetically modified mice with abnormal Treg cells representation [25]. Furthermore, this panel may be instrumental in identifying hitherto overlooked changes in Treg and/or naïve/memory T cell subset cycling in a variety of settings such as vaccination, infection, autoimmunity, and cancer.

## 2 | SIMILARITY TO PUBLISHED OMIPS

The new ground trodden by this OMIP is the examination of cell cycle of naïve/memory $CD4^+$ and $CD8^+$ T cell subsets and of Treg cells by Ki-67/DNA dual staining, with no similarities to other OMIPs.

OMIP-031 and -032 examined naïve/memory T cells, with different purposes. OMIP-031 used a combination of CD44, CD62L, CD27, CD45RA for T cell subset definition, plus a panel of activation and exhaustion markers, with the aim to analyze inhibitor checkpoint expression. OMIP-032 was designed for assessing innate and adaptive immune subsets from mouse organs, including naïve/memory T cell subsets, that were identified based on CD44 and CD62L expression. OMIP-032 did not include any analysis of proliferation or cell cycle.

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### CONFLICT OF INTEREST

A.C.H. is a board member and equity holder in ImmunoQure, AG., and Gamma Delta Therapeutics, and is an equity holder in Adaptate Biotherapeutics.

### AUTHOR CONTRIBUTIONS

**Ambra Natalini:** Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); writing – review and editing (supporting). **Sonia Simonetti:** Conceptualization (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Gabriele Favaretto:** Data curation (supporting); formal analysis (supporting); methodology (supporting); writing – review and editing (supporting). **Giovanna Peruzzi:** Data curation (supporting); formal analysis (supporting); writing – review and editing (supporting). **Fabrizio Antonangeli:** Data curation (supporting); formal analysis (supporting); writing – review and editing (supporting). **Angela Santoni:** Writing – review and editing (supporting). **Miguel Munoz-Ruiz:** Methodology (supporting); writing – review and editing (supporting). **Adrian Hayday:** Supervision (supporting); writing – review and editing (supporting). **Francesca Di Rosa:** Conceptualization (lead); supervision (lead); writing – original draft (lead).

### PEER REVIEW

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## SUPPORTING INFORMATION

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