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OMIP

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

Ambra Natalini[1] | Sonia Simonetti[1,2] | Gabriele Favaretto[1] | Giovanna Peruzzi[3] | Fabrizio Antonangeli[1] | Angela Santoni[2] | Miguel Muñoz-Ruiz[4] | Adrian Hayday[4,5,6] | Francesca Di Rosa[1]

[1]Institute of Molecular Biology and Pathology, National Research Council of Italy (CNR), Rome, Italy

[2]Department of Molecular Medicine, University of Rome "Sapienza", Rome, Italy

[3]Center for Life Nano- & Neuro-Science, Fondazione Istituto Italiano di Tecnologia (IIT), Rome, Italy

[4]Immunosurveillance Laboratory, The Francis Crick Institute, London, UK

[5]Peter Gorer Department of Immunobiology, King's College London, London, UK

[6]National Institute for Health Research (NIHR) Biomedical Research Center (BRC), Guy's and St Thomas' NHS Foundation Trust and King's College London, London, UK

Correspondence
Ambra Natalini, Institute of Molecular Biology and Pathology, National Research Council of Italy (CNR), Viale Regina Elena 291, Rome 00161, Italy.
Email: ambra.natalini@ibpm.cnr.it

Francesca Di Rosa, Institute of Molecular Biology and Pathology, National Research Council of Italy (CNR), Viale Regina Elena 291, Rome 00161, Italy.
Email: francesca.dirosa@cnr.it

Present address
Sonia Simonetti, Medical Oncology Department, Campus Bio-Medico University, Rome, Italy

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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

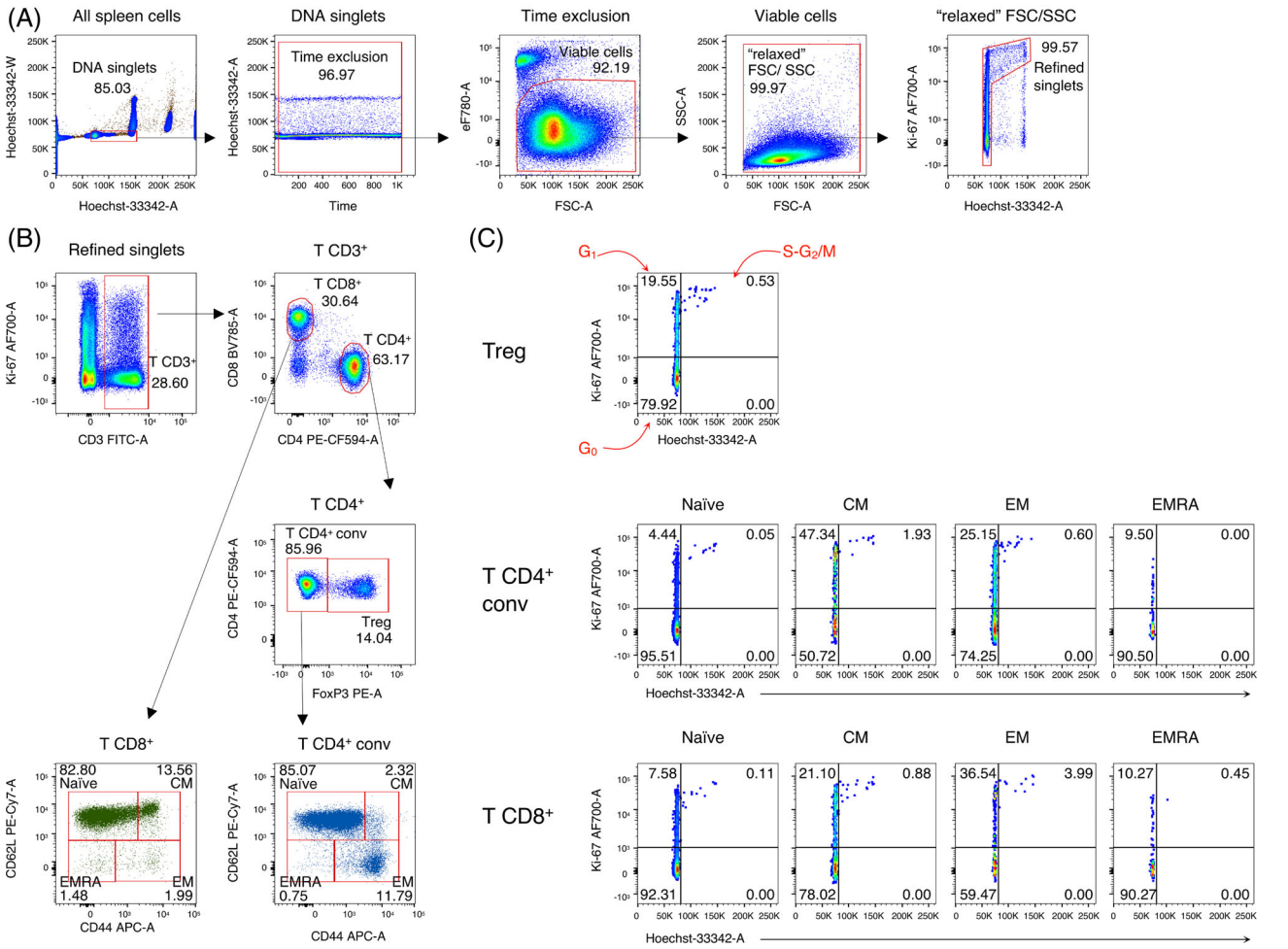

FIGURE 1 Cell cycle analysis of mouse T cell subsets. Example of analysis of spleen cells from a 3-months old C57BL/6J mouse, using manual gating strategy. (A) Refined gating of viable single cells from the spleen in five steps: (1) DNA singlets. Single cells having 2n ≤ DNA content ≤4n were selected 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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ORCID

Francesca Di Rosa https://orcid.org/0000-0003-0252-9138

REFERENCES

1. Farber DL, Netea MG, Radbruch A, Rajewsky K, Zinkernagel RM. Immunological memory: lessons from the past and a look to the future. Nat Rev Immunol. 2016;16:124–8.
2. Di Rosa F. Two niches in the bone marrow: a hypothesis on life-long T cell memory. Trends Immunol. 2016;37:503–12.
3. Tough DF, Sprent J. Turnover of naive- and memory-phenotype T cells. J Exp Med. 1994;179:1127–35.
4. Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J Exp Med. 2007;204:57–63.
5. Rubtsov YP, Niec RE, Josefowicz S, Li L, Darce J, Mathis D, et al. Stability of the regulatory T cell lineage in vivo. Science. 2010;329:1667–71.
6. Sprent J, Surh CD. Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells. Nat Immunol. 2011;131:478–84.
7. White JT, Cross EW, Kedl RM. Antigen-inexperienced memory CD8+ T cells: where they come from and why we need them. Nat Rev Immunol. 2017;17:391–400.
8. Beverley PC. Functional analysis of human T cell subsets defined by CD45 isoform expression. Semin Immunol. 1992;4:35–41.
9. Ivetic A, Hoskins Green HL, Hart SJ. L-selectin: a major regulator of leukocyte adhesion, migration and signaling. Front Immunol. 2019;10:1068.
10. Del Zotto G, Principi E, Antonini F, Baratto S, Panicucci C, Bruno C, et al. Comprehensive phenotyping of peripheral blood T lymphocytes in healthy mice. Cytometry A. 2020;99(3):243–50.
11. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature. 1999;401:708–12.
12. Weninger W, Crowley MA, Manjunath N, von Andrian UH. Migratory properties of naive, effector, and memory CD8(+) T cells. J Exp Med. 2001;194:953–66.
13. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol. 2004;22:745–63.
14. Unsworth A, Anderson R, Haynes N, Britt K. OMIP-032: two multicolor immunophenotyping panels for assessing the innate and adaptive immune cells in the mouse mammary gland. Cytometry A. 2016;89:527–30.
15. Harris NL, Watt V, Ronchese F, Le Gros G. Differential T cell function and fate in lymph node and nonlymphoid tissues. J Exp Med. 2002;195:317–26.
16. Borghans JA, de Boer RJ. Quantification of T-cell dynamics: from telomeres to DNA labeling. Immunol Rev. 2007;216:35–47.
17. Takagi M, Natsume T, Kanemaki MT, Imamoto N. Perichromosomal protein Ki67 supports mitotic chromosome architecture. Genes Cells. 2016;21:1113–24.
18. Cuylen-Haering S, Petrovic M, Hernandez-Armendariz A, Schneider MWG, Samwer M, Blaukopf C, et al. Chromosome clustering by Ki-67 excludes cytoplasm during nuclear assembly. Nature. 2020;587:285–90.

19. Simonetti S, Natalini A, Folgori A, Capone S, Nicosia A, Santoni A, et al. Antigen-specific CD8 T cells in cell cycle circulate in the blood after vaccination. Scand J Immunol. 2019;89:e12735.
20. Muñoz-Ruiz M, Pujol-Autonell I, Rhys H, Long HM, Greco M, Peakman M, et al. Tracking immunodynamics by identification of S-G_2/M-phase T cells in human peripheral blood. J Autoimmun. 2020;112:102466.
21. Ernst DN, Hobbs MV, Torbett BE, Glasebrook AL, Rehse MA, Bottomly K, et al. Differences in the expression profiles of CD45RB, Pgp-1, and 3G11 membrane antigens and in the patterns of lymphokine secretion by splenic CD4+ T cells from young and aged mice. J Immunol. 1990;145:1295–302.
22. Pinchuk LM, Filipov NM. Differential effects of age on circulating and splenic leukocyte populations in C57BL/6 and BALB/c male mice. Immun Ageing. 2008;5:1.
23. Clambey ET, White J, Kappler JW, Marrack P. Identification of two major types of age-associated CD8 clonal expansions with highly divergent properties. Proc Natl Acad Sci U S A. 2008;105:12997–3002.
24. King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell. 2004;117:265–77.
25. Levine AG, Arvey A, Jin W, Rudensky AY. Continuous requirement for the TCR in regulatory T cell function. Nat Immunol. 2014;15:1070–8.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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