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Technical note

An efficient protocol for *in vivo* labeling of proliferating epithelial cells

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ABSTRACT

The study of organogenesis, tissue-homeostasis and regeneration requires the precise assessment of *in vivo* cell proliferation. To this end a host of methods have been developed to detect and quantify DNA synthesis in proliferating cells. These include cell labeling with various nucleotide analogues and fluorescence reporter-based animal models with each method presenting its idiosyncratic shortcomings. Quantitative assessment of epithelial cell turnover has been partly hampered due to their variable and limited *in vivo* accessibility and the requirement for harsher isolation procedures to procure single cells for FACS analysis. Here, we report a reliable protocol to study *in vivo* cell proliferation of epithelial cells in mice by repeatedly injecting EdU intravenously for an extended 12-day period. EdU incorporation was quantitated *ex vivo* by FACS after tissue dissociation in order to obtain single epithelial cell suspensions. As a lead population, we analyzed thymic epithelial cells (TECs), where we were able to label compartmentalized TEC subsets to saturation without apparent toxic effects on the thymus architecture or stress-sensitive TEC lineage differentiation. The data is in concordance with the prevailing model of medullary TEC terminal differentiation that includes the post-Aire stage. The same protocol was successfully applied to epithelial cells of various other organs – skin, lymph node, kidney and small intestine – tissues with widely varying frequencies and rates of proliferating epithelial cells.

1. Introduction

The precise assessment of *in vivo* cell proliferation is essential for understanding organogenesis, tissue homeostasis and recovery. For various reasons, it has been more difficult to efficiently label epithelial cells as compared to hematopoietic cell lineages for *in vivo* cell proliferation studies. Here, we report on an efficient protocol to label epithelial cells. We placed a particular focus on thymic epithelial cells (TECs) that form the structural and functional framework for T cell development and establishment of central T cell tolerance towards self-antigens (Kyewski and Klein, 2006). We chose TECs as a well-studied reference population, which comprise two major lineages – cortical TECs (cTECs) and medullary TECs (mTECs) – whereby the cortex is secluded by a specialized vascular blood-thymus barrier, while the medulla is freely accessible to circulating blood constituents (Raviola and Karnovsky, 1972). Previous studies on TEC turnover and homeostasis using Bromodeoxyuridine (BrdU) yielded partly conflicting results (Gabler et al., 2007; Gray et al., 2007). We re-addressed this issue by efficiently labeling proliferating TECs *in vivo* using the current gold-standard – 5-ethynyl-2′-deoxyuridine (EdU) (Salic and Mitchison, 2008). A wide variety of *in vitro* (Yu et al., 2009; Sun et al., 2012) and *in vivo* studies (Zeng et al., 2010; Ohyama et al., 2013; Amano et al., 2014; Gitlin et al., 2014; Neumann et al., 2014; Fabrice et al., 2015) have shown EdU to have a high resolution, deep diffusion and sample penetration (Chehrehasa et al., 2009). Herein, we describe an efficient protocol by repeatedly injecting intravenously EdU for an extended 12-day period. We thus were able to label all TEC subsets to near saturation without altering the thymus architecture or affecting the most sensitive TEC subset namely the mature Aire$^+$ medullary TECs (mTECs). Importantly, the same protocol could also be successfully applied to epithelial cells of various other organs such as skin, lymph nodes, kidneys and small intestine with widely varying frequencies and rates of proliferating epithelial cells. In all cases, EdU incorporation has been quantitated *ex vivo* by FACS analysis after tissue dissociation and single epithelial cell isolation.

2. Results & discussion

TECs were separated into cTEC and mTEC lineages, and the latter was further sub-divided into immature MHCII^{lo} and mature MHCII^{hi}

Abbreviations: Aire, Autoimmune regulator; BrdU, Bromodeoxyuridine; DP, double positive; EdU, 5-ethynyl-2′-deoxyuridine; LN, lymph node; cTEC, cortical thymic epithelial cell; mTEC, medullary thymic epithelial cell; SI, small intestine

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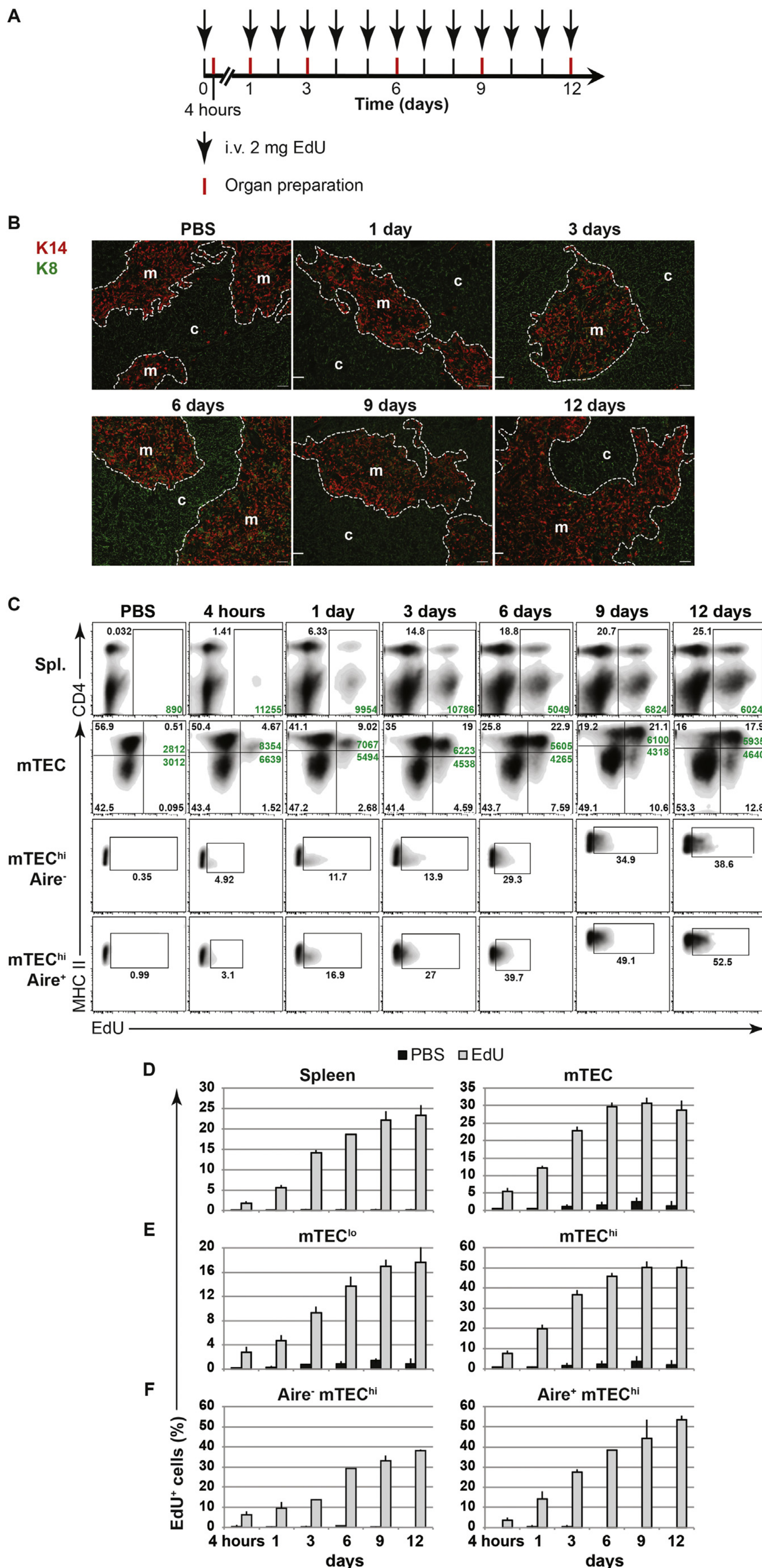

Fig. 1. Analysis of thymus architecture and mTEC turnover upon long-term *in vivo* administration of EdU.

(A) 5–8 week old C57BL/6 mice were injected daily i.v. with 2 mg EdU or PBS for 12 days (shown with a black arrow). Animals were sacrificed 4 h, 1 day, 3 days, 6 days, 9 days or 12 days after the first injection (shown with a red line). (B) Mouse thymi were co-stained with mTEC-specific anti-cytokeratin 14 (red) and anti-cytokeratin 8 (green), which stains the bulk of cTECs, to determine the influence of long-term EdU treatment on thymus integrity. Scale = 50 μm; white dotted line – cortico-medullary junction; c = cortex; m = medulla. (C) Multi-color flow cytometry was used to test the efficacy of EdU labeling in a long-term experiment by determining steady-state cell division of mTEC (subsets). Some of the data were re-calculated from the TPA – EdU experiments, Michel et al., 2017. Splenocytes (Spl.) were used as a positive control for EdU administration. The complete 12-day kinetic was performed twice; 6–8 C57BL/6 mice of 5–8 weeks of age were used for each time-point. EdU^+ cells of each organ (spleen and thymus) were pre-gated on live cells. MFI values for EdU^+ subsets are shown in green. (D–F) Average percentages of EdU^+ splenocytes and mTEC subsets. EdU incorporation in (D) splenocytes (left panel) and mTECs (right panel); (E) Immature $mTEC^{lo}$ (left) and mature $mTEC^{hi}$ (right) and (F) $Aire^-$ (left) and $Aire^+$ (right) $mTEC^{hi}$ subsets. Shown are the standard deviations of two experiments. Note the different scales on the Y-axis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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