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Development of a Mature B Lymphocyte Probe through Gating-Oriented Live-Cell Distinction (GOLD) and Selective Imaging of Topical Spleen

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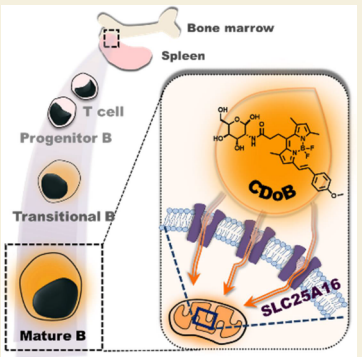
ABSTRACT: B lymphocytes play a pivotal role in the adaptive immune system by facilitating antibody production. Young B cell progenitors originate in the bone marrow and migrate to the spleen for antigen-dependent maturation, leading to the development of diverse B cell subtypes. Thus, tracking B cell trajectories through cell type distinction is essential for an appropriate checkpoint assessment. Despite its significance, monitoring specific B cell subclasses in live states has been hindered by a lack of suitable molecular tools. In this study, we introduce **CDoB** as the first mature B cell-selective probe, enabling real-time discrimination of three classified stages in B-cell development: progenitor, transitional, and mature B cells, through a single analysis using CyTOF. The selective mechanism of **CDoB**, elucidated as gating-oriented live-cell distinction (GOLD), targets SLC25A16, identified through systematic screening of SLC-CRISPRa and CRISPRi libraries. **CDoB** selectively brightens mature B cells in the mitochondrial area using SLC25A16 as the main gate, and the staining intensity correlates positively with the expression level of SLC25A16 along the B cell maturation continuum. In spleen tissues, **CDoB** demonstrates selective marking in mature B cell areas in live tissue status, representing the first performance achieved by a small-molecule fluorescent probe.



KEYWORDS: *fluorescent carbohydrate library, high-throughput phenotypic screening, gating-oriented live-cell distinction, solute carrier, mature B cell specific probe, spleen topical imaging*

INTRODUCTION

The main players in the adaptive immune system are T and B lymphocytes. While T lymphocytes participate in cytotoxic responses, B cells are responsible for antibody production. Given the similarity in shape and size between T and B lymphocytes, it has long been believed that discriminating between the two cell types is practically impossible without the aid of fluorescently labeled antibodies targeting cell surface biomarkers such as cluster of differentiation (CD) markers. However, our research has demonstrated that the development of small molecule fluorescent probes for cell-selective distinction is feasible through the diversity-oriented fluorescence library (DOFL). With these fluorescent probes, we can not only monitor dynamic cellular states but also uncover intrinsic cellular activities without disturbance.[1]

We have developed two B cell selective probes **CDgB**[2] and **CDyB**,[3] which exhibit specificity over T cells. While we achieved general selectivity for B lymphocytes, we recognized the need to differentiate specific B cell subsets. This consideration arises from the potential for particular B cell subclasses to serve as useful indicators for evaluating histopathology based on cellular compartments or formations, such as in the spleen (SP).[4] Furthermore, tracking B cell trajectories is deemed crucial for proper checkpoint assessment during maturation.[5] Consequently, our focus has shifted toward further discriminating B cell subsets throughout their developmental stages.

B cell precursors differentiate from hematopoietic cells in the bone marrow (BM) (Figure S1). Following the early stage of progenitor B cells (pro-B, pre-B, and immature B cells), they migrate to the SP for antigen sensing and further maturation. Immature B cells settle into transitional type 1 B (T1 B) and type 2 B cells (T2 B) successively before maturing into Follicular (FO) B cells and marginal zone (MZ) B cells.[6] A portion of matured B cells may return to the BM as recirculating B cells.[7] Thus, B cells can be classified into three main subgroups: progenitor (pro-B, pre-B, and immature B cells), transitional (T1 and T2 B cells), and mature (FO,

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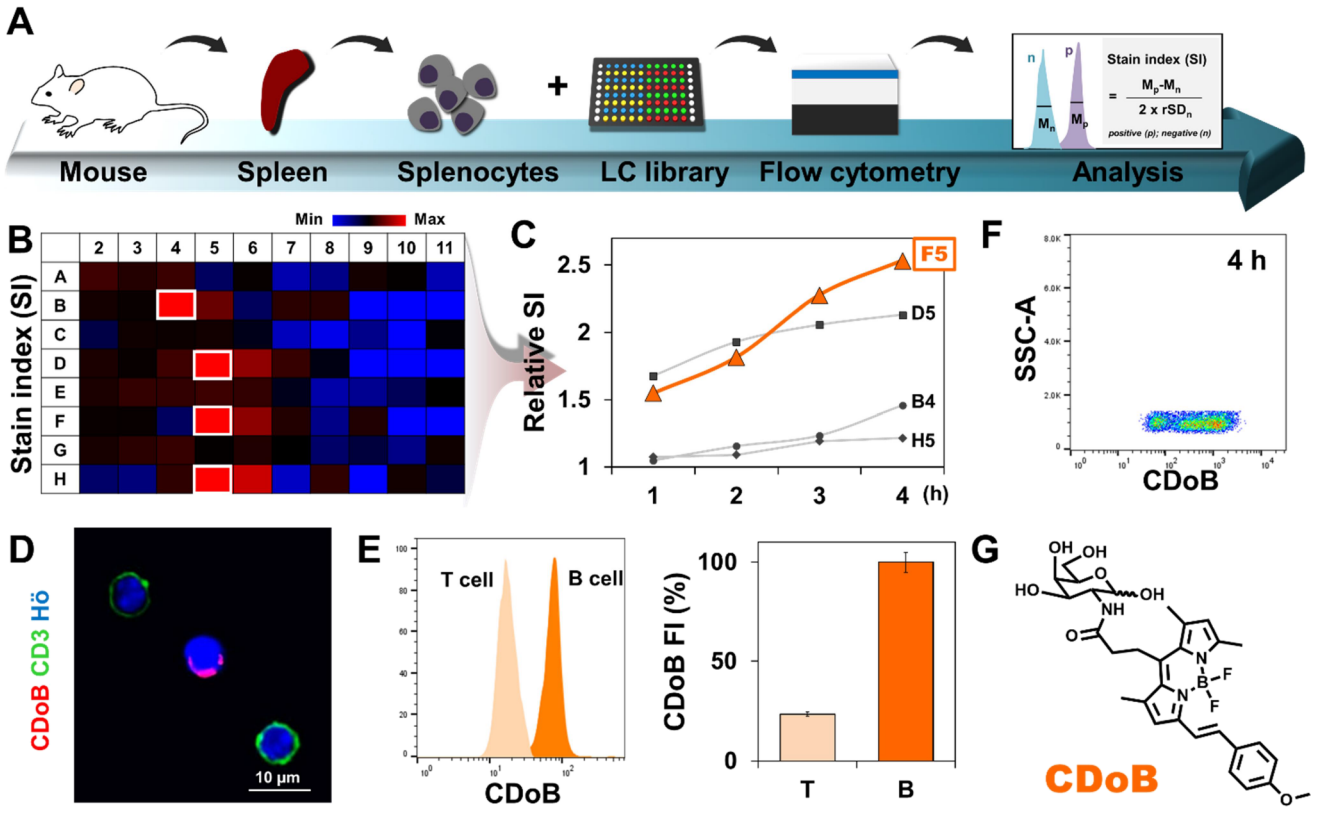

Figure 1. Development of a mouse B cell selective probe. (A) Flowchart of screening with the LC library. (B) Heat map of the stain index (SI) is based on screening results. (C) Deep investigation of behaviors of lead compounds by tracking for 4 h. (D) Total splenocytes were first stained with anti-CD3 (T cell) and then **CDoB** for 1 h. (E) T and B cells from splenocytes were isolated using MACS (magnetic-activated cell sorting). Each cell type was stained with **CDoB** for 1 h and then read with flow cytometry. (F) Three splits were observed after 4 h of staining with **CDoB**. (G) Chemical structure of **CDoB**. The images were taken by a 100 objective oil lens. P, positive; N, negative; M, median fluorescent intensity at half-height; rSD_n, robust standard deviation of negative at half-height. Circle (●), LC-B4; square (■), LC-D5; triangle (▲), LC-F5; diamond (◆), LC-H5.

MZ, recirculating B cells), depending on their maturation stages.

To elicit a more specific B cell probe, we introduced the luminescent-carbohydrate (LC) library to the SP. This approach expands the utility of carbohydrate bioprobes beyond cancer cells, which have notably increased uptake rates of carbohydrates,[8] to image specific immune cell types such as macrophage subsets[9] beyond cancer cells that have striking increased uptake rates of carbohydrates.

In this study, we introduce the first mature B cell-selective probe, **CDoB** (compound designation "orange" or "old" B cell), capable of monitoring the B cell maturation process based on its intensity. Through the utilization of both the SLC-CRISPRa and CRISPRi screening systems, we identified the specific transporter, SLC25A16, which is overexpressed in mature B lymphocytes compared with other subtypes. Furthermore, we demonstrated **CDoB**'s ability to selectively enhance the region of mature B lymphocytes in live SP tissues.

RESULTS AND DISCUSSION

Discovery of a Stratified Mouse B Lymphocyte Selective Probe

To discover a specific B cell subset probe from the LC library, we established an efficient screening format[10] utilizing the SP, which primarily comprises B lymphocytes (around ~70%) along with a minor population of T cells (Figure 1A).[11] Initially, the murine SP was dissociated into single cells of splenocytes, and 1 μM LC compounds were added to the cells. After 1 h, the samples were analyzed using flow cytometry (Figure S2 and Table S1). Based on the outcomes, we calculated the SI to assess the degree of separation in the splenocytes and represented the values in a heat map (Figures 1B and S3). We identified four lead compounds that further divided splenocytes into two groups, but unexpectedly, these molecules shared the same fluorophore. Although we investigated whether only the core structure possessed discriminatory capacity, it failed to distinguish the cell populations (Figure S4). Subsequently, we varied the incubation time up to 4 at 1 h intervals (Figures 1C, S5, and S6). Interestingly, the F5 index showed a significantly higher point at 4 h compared to other molecules, demonstrating three distinct cell populations. Intrigued by this unique pattern, F5 was selected for further study. It was observed that F5 exhibited preferential selectivity for B cells over T cells after 1 h (Figure 1D,E). Furthermore, the two brighter populations among the three splits were identified as B lymphocytes (Figures 1F and S7). Since F5 consists of an orange fluorophore attached to galactose at the 2-carbon position, along with comprehensive recognition of B cells (Figure 1G), it was named **CDoB** (compound designation orange B). These results suggest that both the fluorophore and the carbohydrate moiety contribute to its specific selectivity.

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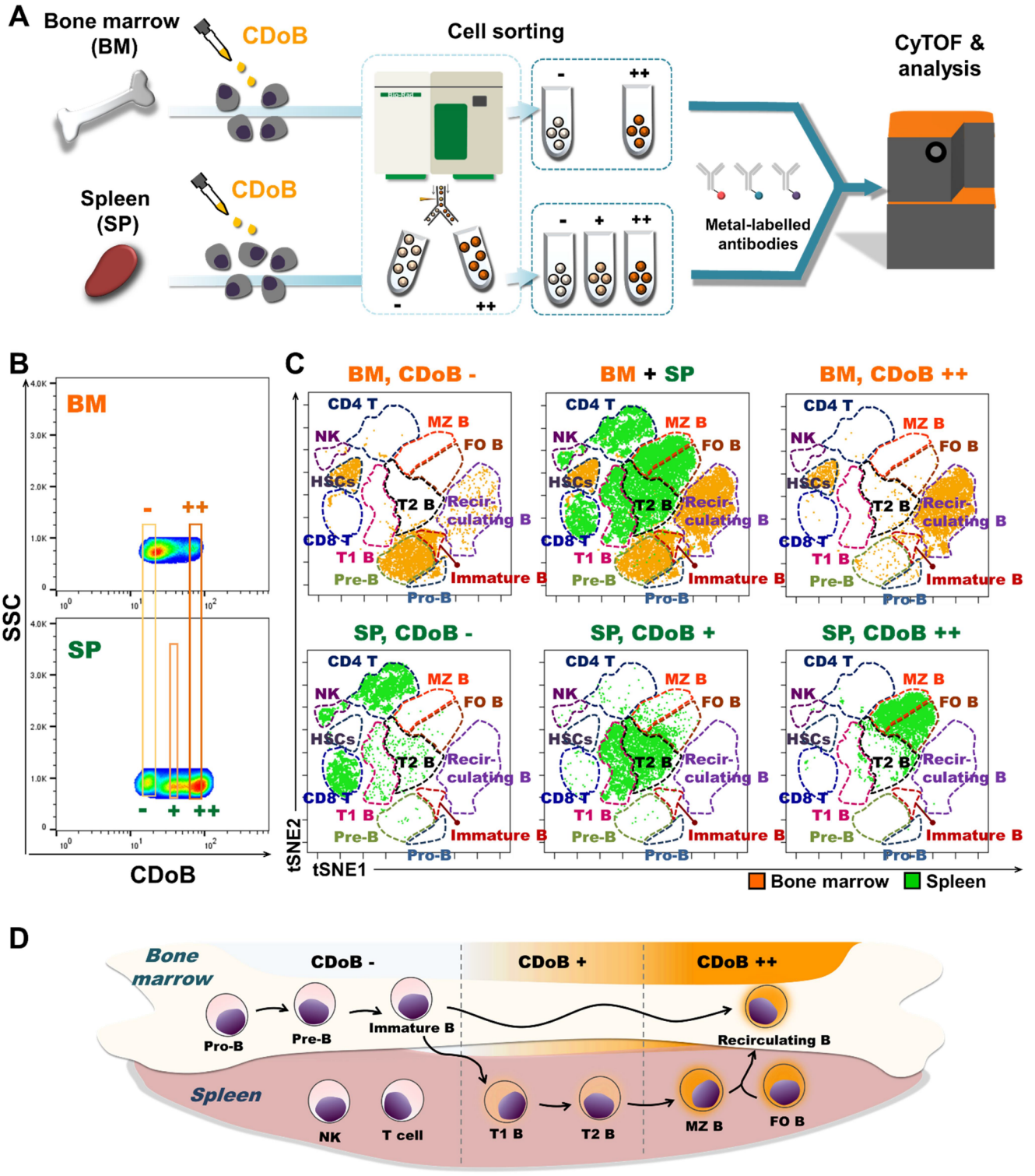

Figure 2. Cell type identification with **CDoB**. (A) Schematic view of experimental procedures. (B) Separation patterns of BM and SP after 4 h of **CDoB** staining. (C) Analysis data of CyTOF after sorting out cells followed by **CDoB** intensity. FO B: follicular B, MZ B: marginal zone B; T1 B: transitional type 1 B. T2 B: transitional type 2 B, HSCs: hematopoietic stem cells. (D) Illustration of **CDoB** selectivity in BM and SP. B cell progenitors (Pro-B, Pre-B, and immature B) in BM are the dimmest along with NK and T cell. These cells immigrated to SP and transferred into transitional B cells (T1 B, T2 B) that showed middle intensity of **CDoB**. Both B cell progenitors and transitional B cells finally developed into mature B cells (MZ B, FO B, and recirculating B) which are the brightest community of **CDoB**.

Cell Type Identification by CDoB Intensity

We identified that **CDoB** has a strong potential to recognize a specific B cell subtype by distinguishing the B cell groups into two subsets. To deeply investigate **CDoB** selectivity, BM and SP were analyzed together, enriching diverse B cell subsets involved in B cell development.

Single cells were initially obtained from BM and SP, respectively, and then **CDoB** was added (Figure 2A). After 4 h,

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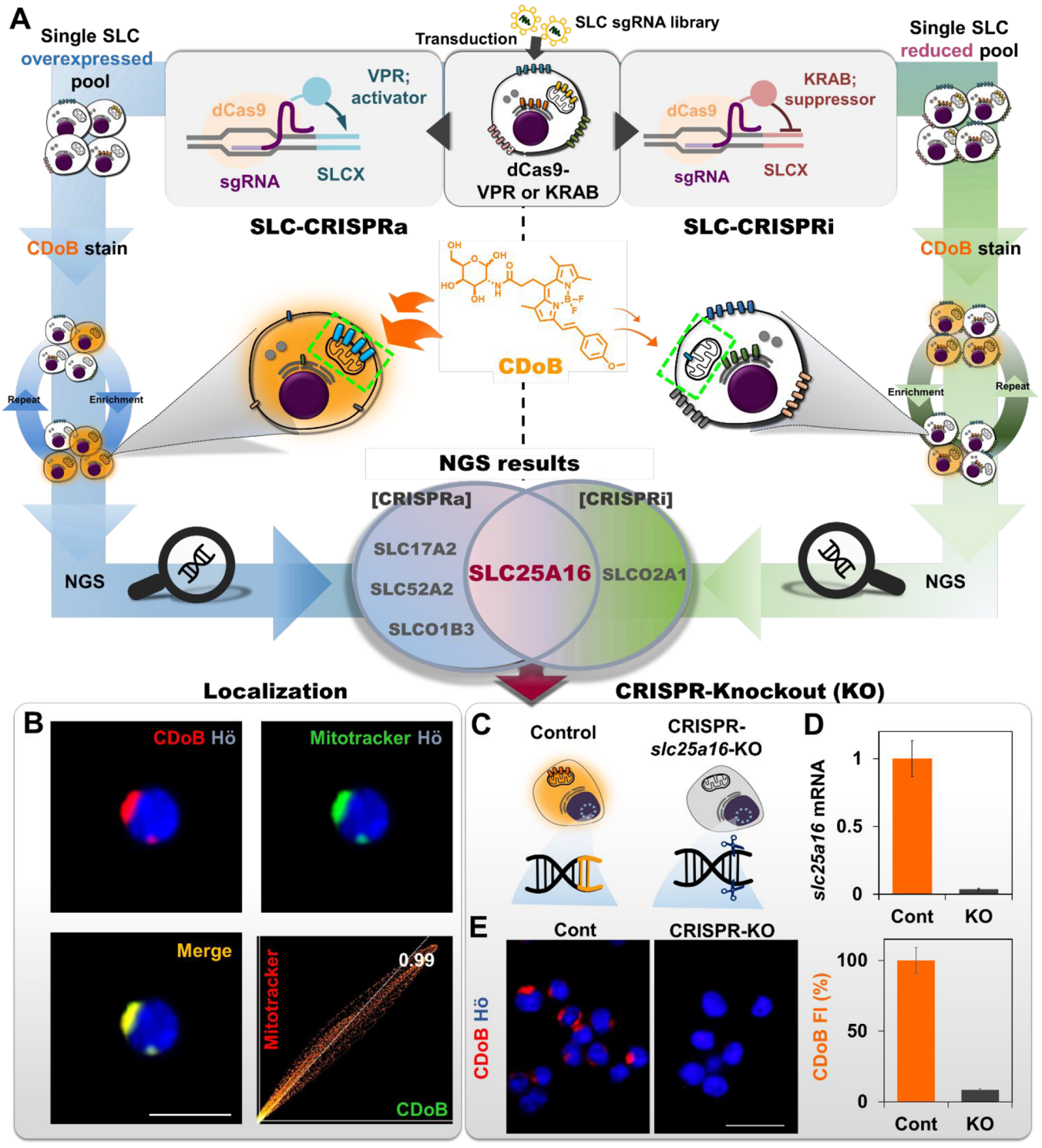

Figure 3. Target identification and validation of CDoB. (A) Schematic process of SLC-CRISPRa and CRISPRi screening and the results. (B) Localization of **CDoB** was confirmed in murine primary B cells, and (F) correlation graph and numeric value of Pearson's correlation coefficient (0.99). ×100 oil objective lens; scale bar: 10 μm. (C) CRISPR-sls25a16-Knockout (KO) was applied to the WEHI-231 cell line. (D) mRNA expression level of *slc25a16* in both control and KO cells. (E) After CRISPR-KO, **CDoB** intensity was diminished in KO cells. ×40 objective lens; scale bar: 20 μm.

we observed distinct staining patterns in BM compared to the SP based on **CDoB** intensity (Figure 2B). While **CDoB** separated the splenocytes into three groups, **CDoB**++ (double positive), + (single positive), and − (negative), only **CDoB**++ and **CDoB**− groups were detected in BM. Subsequent to these findings, we sorted out each of the five groups (two from BM, three from SP). Conventional flow cytometry techniques are inadequate due to limited channels and significant emission spectra overlay.[12] However, cytometry by time of flight (CyTOF) can simultaneously analyze over 90 parameters by targeting cells with a cocktail of antibodies conjugated with heavy metal isotopes. Additionally, CyTOF is effective for

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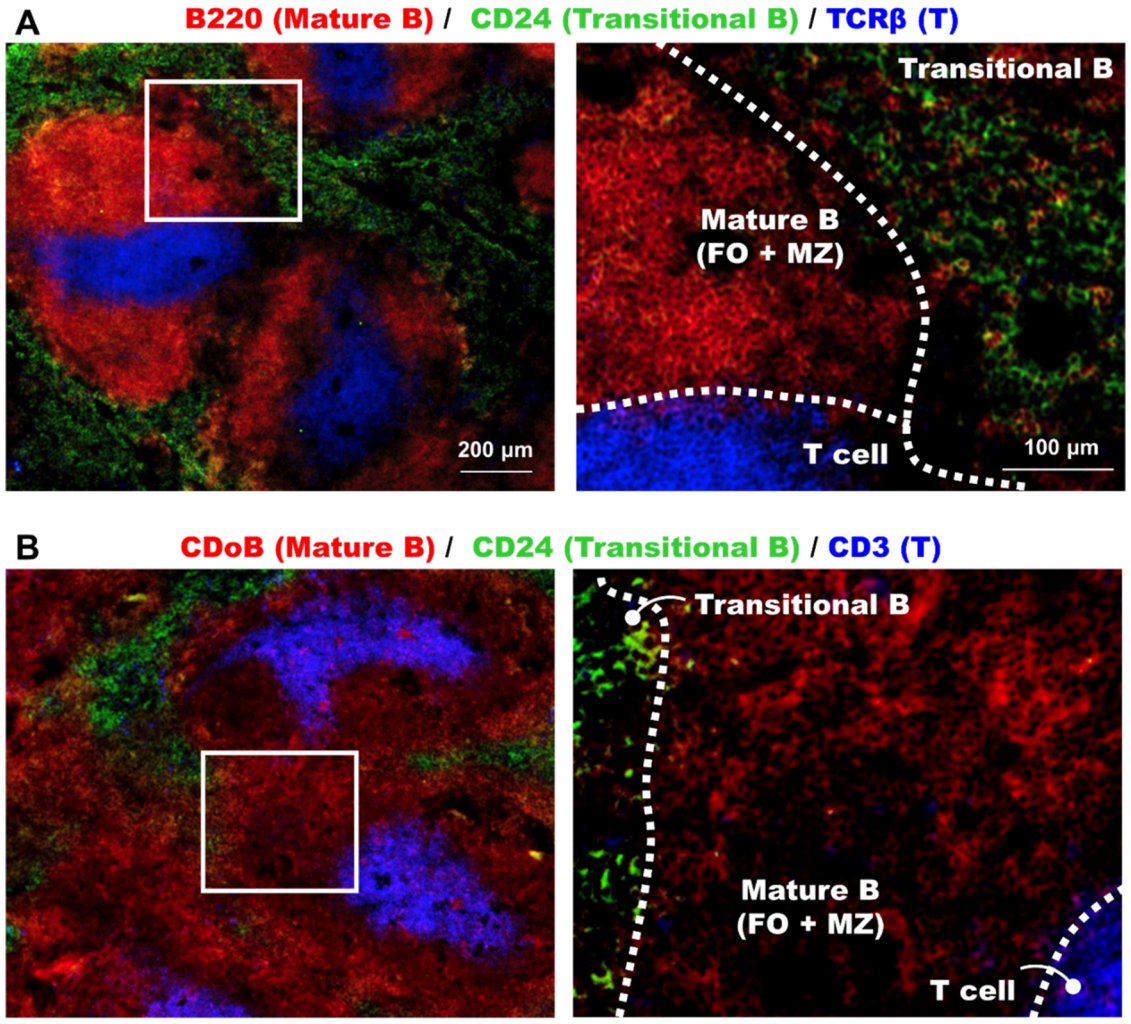

Figure 4. B cell development tracking by CDoB. (A) SP tissues were stained with only antibody. (B) **CDoB** stained the tissue for 4 h, and antibodies were added. The left images were taken by using a 5× objective lens, and the right ones were imaged by a 10× objective lens.

capturing slight variations in biomarker expression in a single analysis, as data from each sample are plotted in a two-dimensional graph concurrently. Leveraging the advantages of CyTOF, we designed antibody panels to encompass B cell subsets in both BM and SP,[13] as well as T and NK cells[14] (Tables S2 and S3). Subsequently, we incubated the sorted cells with metal-labeled antibodies, and the samples were analyzed by CyTOF. The five data sets were combined, generating a two-dimensional map using the t-SNE (t-distributed stochastic linear embedding) algorithm (Figure S8).

The t-SNE maps clustered different phases of B cells along with T lymphocytes and NK cells based on differently expressed antibodies, delineating the lineage of SP (green) and BM (orange) with colored dots (Figure 2C). Interestingly, the results revealed that **CDoB** can monitor B cell developmental stages. The **CDoB**++ populations included mature B cells (FO B, MZ B, recirculating B), whereas **CDoB**+ contained transitional B cells, and **CDoB**− consisted of B cell progenitors (pro-B, pre-B, and immature B), T, and NK cells (Figure 2D). This performance contrasts with previously reported B cell probes, **CDgB**[2] and **CDyB**,[3] which have low sensitivity to monitor overall B cell stages from BM to SP. This result underscores the efficacy of **CDoB** as an ideal tool for tracking B cell maturation, especially in selectively highlighting mature B lymphocytes, leading to its designation as **C**ompound **D**esignation for **o**ld **B**.

Elucidation of the Selectivity Mechanism and the Target Validation of CDoB

Considering the molecular structure and localization of **CDoB**, we hypothesized that **CDoB** may utilize a specific transporter associated with mature B cells. While transporters are broadly categorized into solute carrier (SLC) and ATP-binding cassette (ABC) transporters,[15] SLCs are especially recognized as influx transporters responsible for translocating molecules across cell membranes. With over 400 members and a wide range of selective substrates, the diversity of SLCs may offer insights into **CDoB**'s affinity toward mature B lymphocytes. Therefore, we established a CRISPR-based SLC screening platform (Figure 3A). This system lacked endonuclease activity and controlled target gene expression by introducing effectors: VPR (activation)[16] or KRAB (suppression).[17] We selected approximately 380 protein-encoded SLC genes from the NCBI gene database and designed 10 single guide RNAs (sgRNAs) for each SLC gene with dCas9-VPR, generating 3800 members of the SLC-CRISPRa (activation) library initially.[18] Using the SLC overexpression system, the top 3−5% of the brightest **CDoB** populations were repeatedly sorted and expanded until distinctly bright populations were enriched. After 7 rounds, the

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sorted **CDoB**-bright populations showed significant enrichment compared to control cells (Figure S9). Next-generation sequencing (NGS) analysis revealed four enriched target genes: SLC17A2, SLC52A2, SLCO1B3, and SLC25A16, each appearing in similar proportions (approximately 25%).

To further narrow down the target transporter, we conducted SLC-CRISPRi screening, which suppressed SLC expression using dCas9-KRAB. Unlike SLC-CRISPRa, we sorted out the 3−5% dimest **CDoB** populations and analyzed the sequences from the sorted cells (Figure S10). Interestingly, we identified the overlapping gene, SLC25A16, derived from both CRISPRa and CRISPRi screening analyses. Based on these results, we focused on SLC25A16, known as a mitochondrial carrier,[19] Subsequently, we identified that **CDoB** localizes to mitochondria in B lymphocytes with a high correlation coefficient with Mitotracker (0.99) (Figure 3B). Combining these outcomes, we sought to validate whether **CDoB** utilizes SLC25A16 to localize to the mitochondrial area using the CRISPR-Knockout (KO) technique in the mouse B cell line, WEHI-231 (Figure 3C). After slc25a16 gene deletion (Figure 3D), **CDoB** intensity significantly decreased compared to the control (Figure 3E). Furthermore, we confirmed the expression level of slc25a16 in each of the five sorted populations from BM (**CDoB**++, −) and SP (**CDoB**++, + , −), revealing a correlation with **CDoB** intensity and suggesting SLC25A16 as an indicator in B cell developmental processes (Figures S11 and S12).

This performance elucidated the selective mechanism of **CDoB** through gating-oriented live-cell distinction (GOLD) with SLC25A16 overexpressed in mature B lymphocytes. It marks the first achievement in identifying the molecular target utilizing both SLC-CRISPRa and CRISPRi screening formats. Furthermore, SLC25A16 is considered an orthologue of Leu5p, a yeast protein, which utilizes coenzyme A as a substrate.[20] However, due to the lower homology of the protein between mouse and yeast (approximately 38%), it would be difficult to assume that coenzyme A serves as a substrate for murine SLC25A16.[21] As a result, to the best of our knowledge, **CDoB** represents the first clear substrate identified in murine SLC25A16. We believe that this finding will pave the way for in-depth functional studies of murine SLC25A16.

Visualization of Mature B Cells with CDoB in the SP Tissue

Monitoring immune cells in the SP is crucial, given that the SP is an appropriate organ for assessing histopathology based on its size and cellular compartments.[4] Building on **CDoB** selectivity, we investigated its ability to distinguish mature B cells in the splenic tissue.

First, we confirmed cellular locations in the SP using antibodies. The interior consists of a T cell zone (blue) surrounded by mature B cells (red). Interestingly, the region of transitional B cells (green)[22] was situated outside of mature B cells (Figure 4A). The clearly defined sections reflected that mature B cells (FO B, MZ B) predominantly located in the white pulp, while transitional B cells were found in the red pulp (located outside of the white pulp), which facilitates the transportation of transitional B cells through the bloodstream. After defining the configuration, we stained the samples with **CDoB** and antibodies together (Figures 4B and S13). The results showed that **CDoB** accurately distinguished the territory of mature B cells from T and transitional B lymphocytes. Importantly, we also demonstrated that SLC25A16 expression was upregulated in mature B cells corresponding to the **CDoB** intensity (Figure S14). This result clearly emphasizes not only the selectivity of **CDoB** but also the selectivity of SLC25A16 as a novel biomarker for mature B cells. Moreover, to the best of our knowledge, this is the first case of analyzing the structural splenic tissues with a fluorescent probe, further providing a multidimensional window for cellular analysis.

CONCLUSIONS

In this study, we introduce the mature B cell-selective probe **CDoB**, which effectively discriminates between B cell developmental stages of progenitor, transitional, and mature B lymphocytes based on **CDoB** staining intensity. We elucidated the selective mechanism as GOLD with SLC25A16 overexpressed in mature B lymphocytes through both SLC-CRISPRa and CRISPRi screening. Further validation using the CRISPR-KO technique confirmed that **CDoB** relies on SLC25A16 as the main gate to localize into mitochondria in mature B cells. The function and substrate of SLC25A16 have not been precisely determined, with only a speculative substrate of coenzyme A, observed in yeast protein with lower homology to the murine protein. Therefore, our findings will further lead to a deep investigation of SLC25A16, and its relationship with B cell maturation.

With promising results, we explored the potential of **CDoB** to monitor the topical area of mature B lymphocytes in live splenic tissue. Through its superior performance, we identified SLC25A16 as a potential novel marker for mature B lymphocytes. In summary, we propose that both **CDoB** and SLC25A16 could serve as valuable indicators for monitoring the B cell maturation process. We believe that this research will not only provide a useful index but also shed light on solving complex biological systems using the fluorescent small molecule, **CDoB**.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.4c00001.

Detailed synthetic schemes, experimental procedures, and general information, including NMR spectra; SLC-CRISPR screening and flow cytometry protocols; general information; animal experiments; lymphocyte preparations; flow cytometry-based screening protocol; magnetic-activated cell sorting protocol; image acquisition and localization study; SP tissue imaging protocol; mass cytometry protocol; mass cytometry data analysis method; SLC-CRISPRa/i screening protocols; CRISPR-knockout experiment method; PCR protocols; NMR spectra; and HPLC spectra (PDF)

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Author Contributions

⊥H.C. and H.-Y.K. contributed equally to this work. CRediT: **Heewon Cho** conceptualization, data curation, investigation, methodology, validation, visualization, writing-original draft; **Haw-Young Kwon** conceptualization, funding acquisition, methodology, writing-review & editing; **Youngsook Kim** methodology; **Kyungwon Kim** methodology; **Eun Jig Lee** methodology; **Nam-Young Kang** conceptualization, funding acquisition, methodology, supervision; **Young-Tae Chang** conceptualization, formal analysis, funding acquisition, project administration, supervision, writing-review & editing.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GOLD, gating-oriented live-cell distinction; LC, luminescent-carbohydrate; DOFL, diversity-oriented fluorescence library; CDoB, compound designation orange for B or old B; SLC, solute carrier; ABC, ATP-binding cassette; NGS, next-generation sequencing; BM, bone marrow; SP, spleen; CyTOF, cytometry by time of flight; T1 B, transitional type 1 B; T2 B, transitional type 2 B; FO B, follicular B; MZ B, marginal zone B; sgRNA, single-guide RNA

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