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Rapid capture and labeling of cells on single domain antibodies-functionalized flow cell

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Abstract

Current techniques to characterize leukocyte subgroups in blood require long sample preparation times and sizable sample volumes. A simplified method for leukocyte characterization using smaller blood volumes would thus be useful in diagnostic settings. Here we describe a flow system comprised of two functionalized graphene oxide (GO) surfaces that allow the capture of distinct leukocyte populations from small volumes blood using camelid single-domain antibody fragments (VHHs) as capture agents. We used site-specifically labeled leukocytes to detect and identify cells exposed to fungal challenge. Combining the chemical and optical properties of GO with the versatility of the VHH scaffold in the context of a flow system provides a quick and efficient method for the capture and characterization of functional leukocytes.

Keywords

Single domain antibody; Graphene oxide; Sortase; Enzymatic labeling; Cell detection; Leukocytes

1. Introduction

Peripheral blood represents an attractive source of material for clinical studies, given the minimally invasive procedure required for sample collection and the capacity of such samples to report on perturbations of the immune and inflammatory status. Immunological assays usually involve enumeration and characterization of white blood cells or leukocytes (WBC's). The tracking of cell surface proteins mostly relies on fluorophore-conjugated antibodies or the intrinsic fluorescence provided by fluorescent protein fusions (Bellucci et al., 2015; Kim et al., 2015; Policarpo et al., 2014). We have generated red blood cells (RBCs) that contain modified surface proteins equipped with a sortase tag. The presence of this tag allows covalent installation of a wide range of probes (Li et al., 2015; Shi et al.,

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2014). Despite the development of several methods to label cells from blood (de Vries et al., 2010; Pillay et al., 2010; Salmi and Jalkanen, 2005), none affords selective labeling of specific leukocytes due to the short lifespan of these cells (Kolaczowska and Kubes, 2013). Existing methods to characterize leukocytes *ex vivo* require comparatively long sample preparation times that involve isolation, labeling, and washing steps, all of which may affect the responsiveness of leukocytes (Agrawal et al., 2008). In addition, many leukocyte subtypes share surface markers. For instance, both B cells and monocytes express class II MHC products and similarly both monocytes and neutrophils express CD11b (Hume, 2008; Lai et al., 1998). A fast and reliable method that allows the isolation of specifically labeled leukocytes from blood would thus be useful.

Graphene oxide (GO) provides an attractive option to improve the sensitivity of biosensors (Chen et al., 2012b; Li et al., 2013; Yoon et al., 2013). Typical GO-based biosensors are created by covalently immobilizing antibodies via exposed lysine residues of antibodies to the activated carboxyl group on GO (Jung et al., 2010). However, this method results in random orientation of the antibody. Orienting single domain antigen-binding fragments, also known as VHHs or nanobodies, on sensor surfaces by click chemistry leads to greatly improved sensitivity for biosensors (Trilling et al., 2013, 2014). The small size of VHHs (~15 kDa) and their excellent thermal and chemical stability profile make them suitable for numerous diagnostic and therapeutic applications (De Meyer et al., 2014; Muyldermans, 2013; Siontorou 2013). Since a large percentage of the VHH surface is involved in binding interactions, it is essential to create a uniform orientation using site-specific modifications (Trilling et al., 2013, 2014) to improve the biosensor's performance. To achieve consistency in orientation, we previously used a combination of sortase-mediated trans-peptidation reactions and 'click' chemistry to site-specifically link VHHs with linkers coated onto GO (Agrawal et al., 2008; Chen et al., 2015). The use of these techniques allowed for quick and efficient capture of a distinct leukocyte subpopulation from small volumes of blood.

Here we use transgenic mice that express dectin-1-LPETG-(HA)₃, a sortase-modifiable protein (Jung et al., 2010; Strijbis et al., 2013; Tafesse et al., 2015), on CD11b positive (CD11b⁺) cells. These engineered CD11b⁺ cells can be labeled with a sortase-catalyzed reaction under native conditions (Fig. 1). In this system, blood samples pass over two surfaces functionalized with an anti-murine Class II MHC VHH (VHH7) and an anti-murine CD11b VHH (VHH DC13), respectively. To demonstrate the potential value of this for further diagnostic applications, we also examined whether surfaces functionalized with a VHH can capture labeled blood cells engaged in the phagocytosis of *Candida albicans*.

2. Experimental section

2.1. Preparation of labeled VHH for immobilization

We synthesized the probe used for VHH labeling, GGGK-[TAMRA]-K-N₃, with an N terminal amine and a C terminal CONH₂ following standard solid phase synthesis protocols (Chen et al., 2015). Calcium-independent heptamutant Sortase A (Li et al., 2015) (10 μM final concentration) and probe (1 mM final concentration) were added to the VHH (200 μM final concentration) in PBS at 20 °C for 2 h. Ni-NTA (0.5 ml, Qiagen) was then added to the reaction mixture and mixed for 20 min to remove sortase and any unreacted VHH. The

desired product, the VHH-peptide adduct, was purified by size exclusion chromatography (Superdex 75- GE Life Sciences).

2.2. Immobilization of VHH to GO nanosheets

GO-coated glass slides were activated with 2 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 5 mM N-hydroxysulfo-succinimide (NHS) in 0.1 M MES (2-[morpholino]ethanesulfonic acid, 0.5 M NaCl, pH=6.0) for 15 min at 25 °C. Slides were coupled with 0.5 mM poly(ethylene glycol) bis(amine) (MW 3000) at 25 °C, followed by installation of dibenzocyclooctyne-sulfo-N-hydroxysuccinimidyl ester (DBCO-NHS ester) in PBS for 6 h. Slides were incubated with 50 µM VHH7 or VHH DC13 labeled with peptide GGGK-TAMRA-K-N₃ in PBS for 12 h. The VHH7 and VHH DC13-GO functionalized nanosubstrates were assembled with another glass slide to form a chamber with two different coating surfaces (Agrawal et al., 2008; Chen et al., 2015).

2.3. Generation of dectin-1-LPETG-(HA)₃ transgenic mice

Mice expressing dectin-1-LPETG-(HA)₃ were generated as described by Beard et al. (2006). Briefly, a fragment containing the tagged version of murine dectin-1 cDNA was cloned into the ColA1/neomycin/hygromycin targeting vector, which contains the ColA1-targeting arms. The linearized targeting vector was introduced into B6;129 ES cells by electroporation and cells were selected with G418. Selected clones were checked for the desired insertion by PCR using the primers: 5'-GCACAGCATTGCGGACATGC-3', 5'-CCCTCCATGTGTGACCAAGG-3' and 5'-GCAGAAGCGCGGCCGTCTGG-3'. The ES cells were injected into a blastocyst which was then implanted into pseudopregnant females to obtain chimeras. The chimeric animals were crossed with C57BL/6 wild-type animals. Those showing germline transmission of the modified dectin construct were selected. The mice were then crossed to B6.129P2-Lyz2tm1(cre)Ifo/J animals (Jackson laboratory) to express the transgene exclusively in lysozyme positive cells. To eliminate expression of endogenous dectin-1, the animals were further crossed with dectin-1-deficient mice kindly provided by Dr. Stuart Levitz and Dr. Gordon D. Brown (Jung et al., 2010; Saijo et al., 2007). These mutant mice were then bred to obtain homozygous for sortagable dectin-1.

2.4. Immunofluorescence microscopy

To capture cells from whole blood, samples (30 µL) were obtained from wild type (WT) or transgenic mice and collected in heparinized tubes. These samples were then loaded into the VHH-functionalized flow system for cell capture. After 3 washes with PBS, cells captured on the VHH DC13-immobilized substrate were fixed with 4% paraformaldehyde solution in PBS, followed by a PBS wash and staining with Alexa647-conjugated anti-mouse CD11b (101218, Biolegend), Alexa647-conjugated anti-mouse Gr-1 (108418, Biolegend) or Alexa647-conjugated anti-mouse HA-tag (3444, Cell Signaling Technology) antibody (1:1000 dilution) by incubation for 1 h at 25 °C. Cells captured on the VHH7-immobilized substrate were fixed and incubated with anti-mouse IgG Alexa568-conjugated antibody (A-11031, Life Technologies) for 1 h at 25 °C in the dark. After a wash with PBS, captured cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Vector Labs) and visualized by confocal microscopy. Images were captured and analyzed using Volocity software (PerkinElmer).

2.5. Fluorescence-activated cell sorting (FACS) analysis of peripheral blood leukocytes

Peripheral blood was collected from both wild-type and dectin-1-LPETG-(HA)₃ transgenic animals. After lysis of red blood cells, freshly isolated leukocytes were stained with an anti-HA-tag antibody (D5-1722, Columbia Biosciences) conjugated with PE. Cells were analyzed by FACS and different white blood cell types were discriminated by their SSC and FSC profile. Modified dectin-1-LPETG-(HA)₃ was detected at the cell surface using anti-HA antibody.

2.6. Sortase labeling

To label cells expressing dectin-1-LPETG-(HA)₃, 30 μ L of 50 μ M sortase A (sortase) and 500 μ M GGGC-Alexa647 peptide were pre-incubated in PBS on ice for 15 min, followed by mixing with 60 μ L of blood from either wild type or transgenic animals. Mixtures were incubated on ice for 60 min with occasional gentle mixing. The mixture was loaded into the VHH-functionalized flow chamber for cell capture. After a wash with PBS, cells were fixed and stained with DyLight550-conjugated anti HA-tag antibody (ab117513, Abcam) or DAPI and visualized by confocal microscopy.

2.7. Capture of labeled cells from whole blood engaging in phagocytosis of *Candida albicans*

Fresh blood from transgenic mice was incubated for 30 min at 37 °C with *Candida albicans* that express blue fluorescent protein (*Candica*-BFP) (Strijbis et al., 2013), followed by incubation with GGGC-Alexa647 peptide and sortase to label dectin-1-LPETG-(HA)₃ positive cells. The sample was then loaded into the VHH-functionalized flow chamber for cell capture. Cells were fixed and visualized by confocal microscopy.

3. Results and discussion

3.1. Principle

Surfaces functionalized with VHH7 and VHH DC13 can selectively capture Class II MHC-positive (MHC⁺) and CD11b⁺ cells from small volumes of peripheral blood (Agrawal et al., 2008; Chen et al., 2015). We now show that this device can also capture primary cells from dectin-1-LPETG-(HA)₃ transgenic mice. GO was immobilized on a 3-APTES-functionized glass slide through electrostatic forces between the oxide groups of GO and the amine-end of 3-APTES (Chen et al., 2012a). To enable conjugation with a dibenzocyclooctyl (DBCO) click partner localized to the GO nanosheet, we engineered a sortase-ready version of VHH7 and VHH DC13 with an LPETG motif near the C-terminus. A Gly₃ peptide containing a TAMRA fluorophore and an azide to enable coupling with DBCO during the 'click' reaction was introduced using standard sortagging protocols (Witte et al., 2012). The TAMRA fluorophore was included to verify protein immobilization (Fig. S1a). The modified VHH7 and VHH DC13 VHH were thus covalently linked to GO nanosheet in a uniform manner (Fig. S1b). The VHH7 and VHH DC13-GO functionalized nanosubstrates were assembled with an additional glass slide to form a cell capture chamber (Fig. S2a). Two pieces of double sided tape were placed on the side of the empty glass slide before pressing onto VHH7 and VHH DC13-immobilized substrates to generate two adjacent chambers of overall

12 mm×50 mm size. The cell capture chamber has a height of 0.1 mm and each therefore contains ~30 μ L of fluid. Other cell-capture devices, such as antibody-coated bead-based pull down assays and flow cytometry-based cell sorting, require longer sample preparation times and usually larger sample volumes (Li and Kurlander, 2010; Zhang et al., 2008). This VHH-based device allows quick and efficient capture of distinct leukocyte subpopulations from small blood sample volumes.

3.2. Capture of cells from transgenic mice

Dectin-1 is a major β -glucan receptor on neutrophils and monocytes (CD11b⁺ cells) and plays an important role in the phagocytosis of *Candida albicans* (Branzk et al., 2014; Esteban et al., 2011; Strijbis et al., 2013). The use of a site-specific labeling method has allowed us to monitor the behavior of fluorophore-labeled functional dectin-1 on the cell surface (Esteban et al., 2011; Strijbis et al., 2013). However, since most dectin-1 positive neutrophils have a short lifespan (Kolaczowska and Kubes, 2013), it is difficult to image labeled leukocytes from whole blood if long isolation and processing times are involved. We first tested the performance of two VHH-modified substrates for rapid capture of CD11b⁺ cells from transgenic mice that express engineered sortase-ready dectin-1. Fresh peripheral blood samples were obtained from transgenic mice that express dectin-1-LPETG-(HA)₃ on the surface of neutrophils and other lysozyme-positive leukocytes (Esteban et al., 2011; Kirak et al., 2010; Shi et al., 2014; Strijbis et al., 2013) (Fig. 2a). The expression of the endogenous untagged version of dectin-1 was eliminated by crossing these animals with dectin-1 deficient mice. Flow cytometry analysis of leukocytes from whole blood collected from the transgenic animals showed that ~11–20% of total granulocytes and monocytes are HA-tagged (Figs. 2b and S3), with tagged cells representing ~1–2% of the total cell population (Fig. S3). These experiments confirm the expression of dectin-1-LPETG-(HA)₃ specifically in lysozyme-positive cells such as granulocytes and monocytes (i.e. CD11b⁺ cells). We next evaluated the potential of the VHH-functionalized flow cell to capture dectin-1-LPETG-(HA)₃ cells from the transgenic mice. Class II MHC⁺ cells (mostly B cells) can be depleted by first passing the sample through the VHH7-coated surface (Fig. S4), thus improving the capture specificity of CD11b⁺ cells on the second VHH DC13-modified substrate (Agrawal et al., 2008; Chen et al., 2015). Confocal microscopy showed that a large proportion (~96%) of CD11b⁺ cells from transgenic mice were retained on VHH DC13-modified nanosubstrates (Figs. 2c and S5). Most of the captured CD11b⁺ cells contain multilobular nuclei, a defining characteristic of neutrophils (Branzk et al., 2014; Kolaczowska and Kubes, 2013). The identification of this population was further confirmed by cell-surface staining with Alexa647-conjugated anti Gr-1 (a surface marker for neutrophils) (Fig. S6). In addition, a fraction of the captured CD11b⁺ cells was stained with Alexa647-conjugated anti HA-tag antibody (Fig. 2d), thereby confirming that the VHH-functionalized flow cell can capture and identify dectin-1-LPETG-(HA)₃ cells.

3.3. Capture of sortase-labeled cells from transgenic mice

To determine whether the C terminus of dectin-1-LPETG-(HA)₃ can be labeled on intact blood cells, GGGC-Alexa647 peptide (500 μ M) with or without sortase (50 μ M) was gently mixed with blood and incubated on ice for 60 min (Fig. 3a). Sortase A acts as a transpeptidase which recognizes the LPETG motif and cleaves between the Thr and Gly

residues, forming a thioester intermediate. An N-terminal poly-glycine-based peptide then serves as nucleophile to resolve the thioester to form the final ligated product, which contains a natural amide bond linkage in the peptide backbone (Popp et al., 2007; Popp and Ploegh, 2011; Tsukiji and Nagamune 2009). Here we use GGGC-Alexa647 peptide as the nucleophile to selectively label the C terminal LPETG of dectin-1 prior to capturing the desired cell population with our VHH-functionalized flow cell. (Fig. 3b). Blood from wild type mice was used as a control, and all captured cells were stained with DAPI. Confocal microscopy images revealed the surface of sortase-labeled cells exhibited a clear fluorophore signal when compared with cells not exposed to sortase or with the wild type control. Labeled cells captured on the substrates were stained with DyLight 550-conjugated anti-HA antibody, which showed a decrease in signal intensity resulting from loss of the HA tag upon sortase labeling (Fig. S5). The flow cell is thus able to efficiently capture labeled cells of low abundance in peripheral blood and enables the analysis of individual captured cells via immunofluorescence staining.

3.4. Capture of labeled cells binding to *Candida albicans*

To assess the potential of our device to capture labeled cells exposed to a fungal challenge, fresh blood from transgenic mice was first incubated with *Candida albicans* for 30 min at 37 °C. A strain of *Candida* engineered to express blue fluorescent protein (*Candida*-BFP) was used. After incubation, labeled cells were loaded into the VHH-functionalized chamber. The captured cells were then fixed and stained using Alexa647-conjugated anti-CD11b. Merged confocal images were used to analyze the percentage of CD11b⁺ cells that bound *Candida albicans*, as shown in Fig. 4a. Confocal microscopy images showed that all captured CD11b⁺ cells were in the process of engulfing *Candida albicans*. The process of *Candida albicans* phagocytosis is not limited to the small percentage of dectin-1 positive cells detected using sortase-labeling (Fig. 3b). Yeast recognition and phagocytosis in dectin-1 deficient cells can occur through the interaction of the yeast with other receptors, such as mannose or C3 receptors (Li et al., 2011; McDonald et al., 2012; van de Veerdonk et al., 2009).

We next mixed a blood sample pre-incubated with *Candida albicans*, with sortase and GGGC-Alexa647 peptide to label dectin-1 positive cells with the fluorophore. The mixture was then loaded into two VHH-functionalized flow cells and visualized by confocal microscopy. Confocal microscopy images revealed that Alexa647 positive cells in the process of engulfing *Candida albicans* had been captured in the VHH-functionalized flow cell. The surface of these cells exhibited red fluorescence from the Alexa647 fluorophore, while control cells incubated without sortase did not show fluorescence (Fig. 4b). The VHH-functionalized flow system thus provides a convenient and reliable method to directly measure and track pathogen-exposed effector cells in the blood without any immunofluorescence staining.

4. Conclusions

We have shown an efficient site-specific labeling of cell surface receptors on intact cells using sortase (Popp and Ploegh, 2011; Witte et al., 2013). VHH-immobilized GO flow cells

efficiently capture labeled leukocytes from small amounts of blood, allowing the detection of cells exposed to a fungal challenge in a timely manner. This method can be potentially applied to studies of immune homeostasis, leukocyte trafficking and fungal recognition from whole blood samples. Finally, the implementation of a VHH-based sensing system has the advantage of small sample volumes, rapid processing, high stability and low cost. This method holds promise for the isolation, capture, labeling, identification and analysis of other cell types, such as different leukocytes, in a minimally invasive detection and diagnostics format.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2016.10.015>.

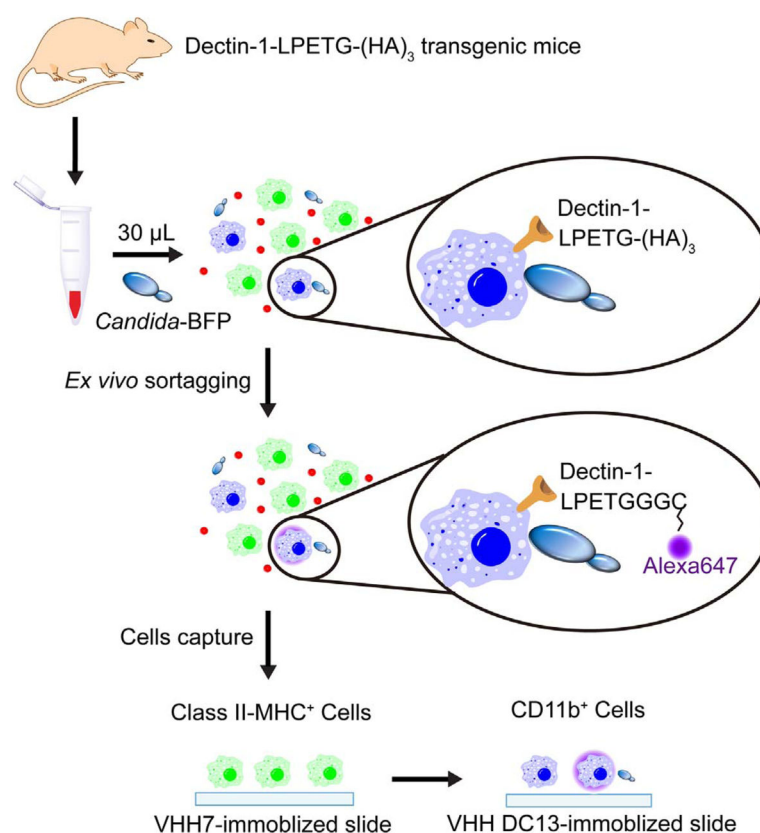
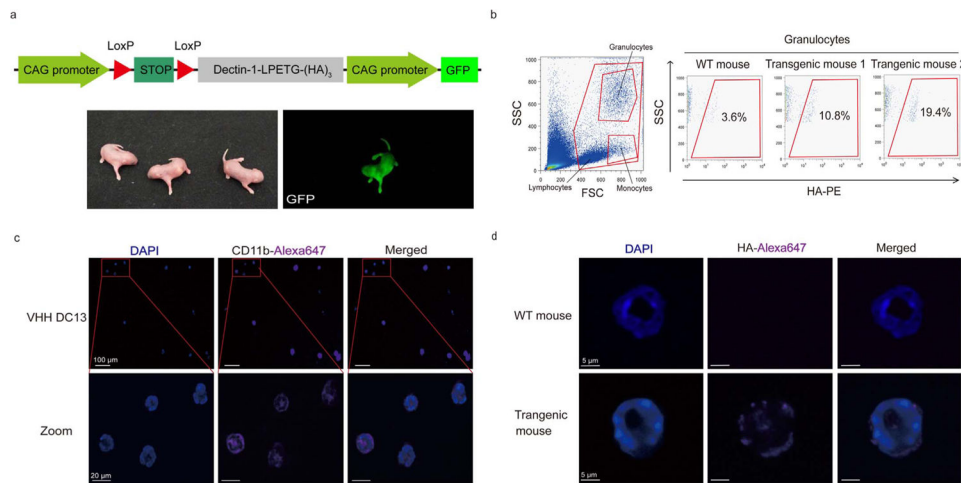
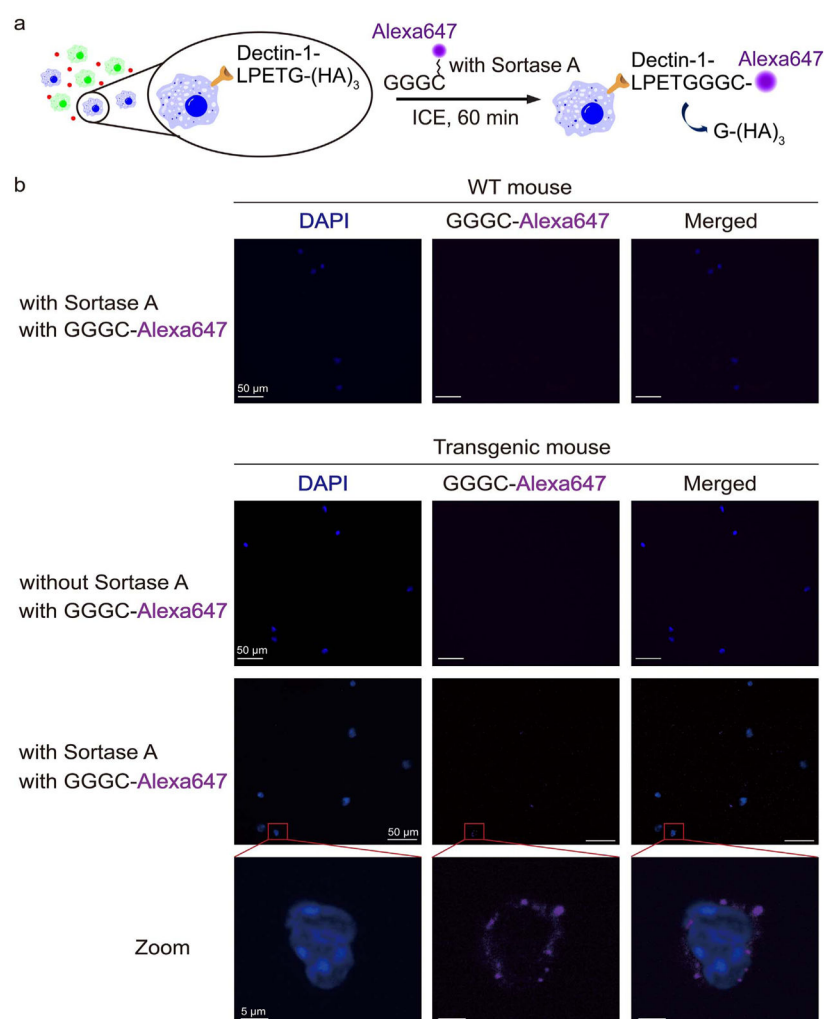


Fig. 1. Schematic illustration of VHH-functionalized flow cell for capture of sortase-labeled cells from small blood sample volumes.

**Fig. 2.**

Whole blood analysis from dectin-1-LPETG-(HA)₃ transgenic mice. (A) Generation of dectin-1-LPETG-(HA)₃ transgenic mice. (B) FACS analysis of murine peripheral blood granulocytes expressing dectin-1-LPETG-(HA)₃. (C–D) Confocal microscopy images of captured cells from transgenic mice after further staining with DAPI, Alexa647-labeled anti-CD11b and Alexa647-labeled anti-HA-tag antibody. A small volume of blood (30 μL) from transgenic mice was loaded into the VHH-functionalized flow cell and incubated at room temperature for 10 min. The chamber was then disassembled and the cell-bearing VHH DC13-immobilized substrate slide was gently washed 3× with PBS. After washing, cells were analyzed by confocal microscopy.

**Fig. 3.**

Isolation of sortase-labeled cells from 30 μ L of whole blood. (A) Sortase-mediated labeling of cells expressing dectin-1-LPETG-(HA)₃. (B) Confocal microscopy images of dectin-1-LPETG-(HA)₃ cells from transgenic mice with or without sortase-mediated labeling. Sortase-labeled cells with dectin-1-LPETGGGC-Alexa647 were captured on VHH DC13-modified substrate. A blood sample from a wild-type (WT) mouse served as the control.

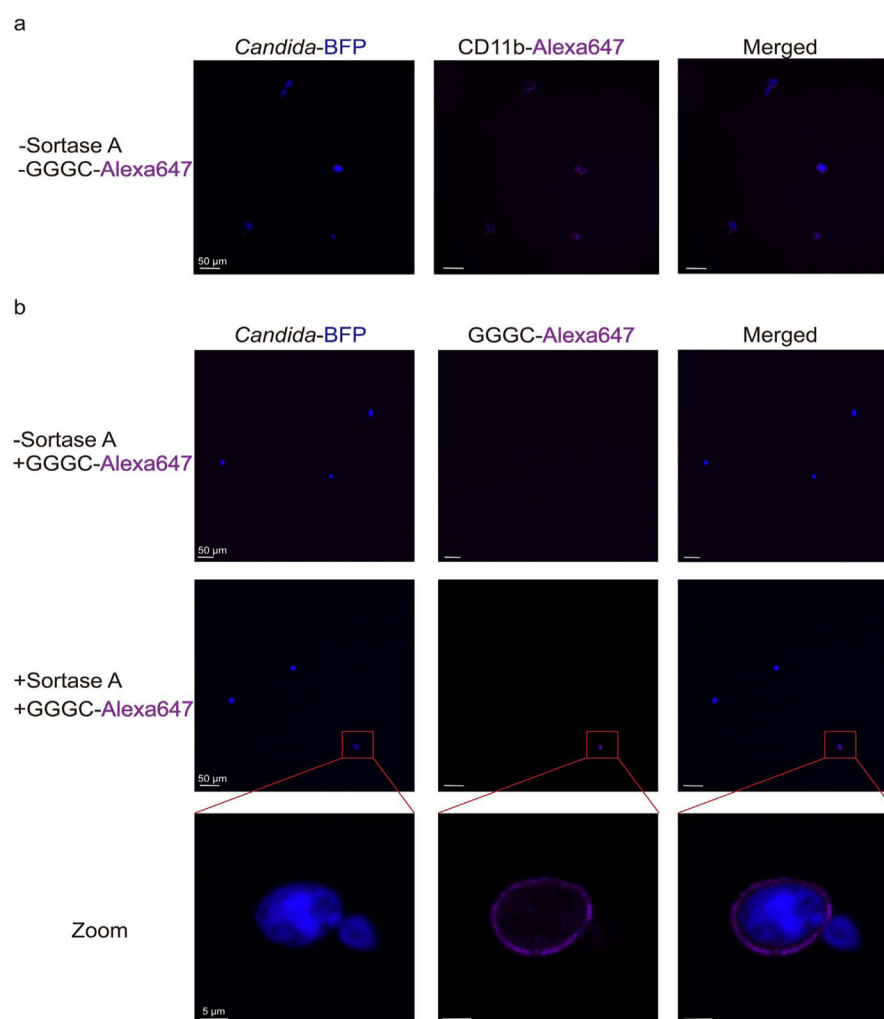


Fig. 4. Combination of VHH7 and VHH DC13-modified nanosubstrates allows identification of labeled cells in the process of phagocytosing *Candida albicans*. (A) Confocal microscopy shows CD11b⁺ cells captured on VHH DC13-modified substrates, dectin-1-LPETG-(HA)₃ in the process of phagocytosing *Candida albicans*-BFP (*Candida*-BFP). (B) Fresh blood from transgenic mice was incubated with *Candida albicans*-BFP (*Candida*-BFP) for 30 min, followed by sortase-mediated labeling as described. Confocal microscopy shows labeled cells that acquired *Candida albicans* phagocytosis, captured after passing through the VHH-functionalized flow cell.