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Research paper

Visualization of integrin Mac-1 *in vivo*

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ABSTRACT

β2 integrins play critical roles in migration of immune cells and in the interaction with other cells, pathogens, and the extracellular matrix. Among the β2 integrins, Mac-1 (Macrophage antigen-1), composed of CD11b and CD18, is mainly expressed in innate immune cells and plays a major role in cell migration and trafficking. In order to image Mac-1-expressing cells both in live cells and mouse, we generated a knock-in (KI) mouse strain expressing CD11b conjugated with monomeric yellow fluorescent protein (mYFP). Expression of CD11b-mYFP protein was confirmed by Western blot and silver staining of CD11b-immunoprecipitates and total cell lysates from the mouse splenocytes. Mac-1-mediated functions of the KI neutrophils were comparable with those in WT cells. The fluorescence intensity of CD11b-mYFP was sufficient to image CD11b expressing cells in live mice using intravital two-photon microscopy. *In vitro*, dynamic changes in the intracellular localization of CD11b molecules could be measured by epifluorescent microscopy. Finally, CD11b-expressing immune cells from tissue were easily detected by flow cytometry without anti-CD11b antibody staining.

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1. Introduction

Leukocytes circulate through blood vessels and lymphoid organs during normal condition and infiltrate the inflamed tissue upon infection or tissue damage. These processes are mediated by the dynamic interaction of integrins with their ligands, being governed by signals from various chemokines presented in the local tissues (Schall and Bacon, 1994; Klaus et al., 2007). Integrins, heterodimers of α- and β-subunits, are a large family of cell surface receptors that are responsible for cell adhesion and migration (Hynes, 2002). Among the three β integrin families (β1, β2 and β3), the β2 integrin family includes LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), the major integrins responsible for leukocyte trafficking (Hyun et al., 2009). Mac-1 is essential for intravascular crawling of innate immune cells such as neutrophils and monocytes (Phillipson et al., 2006; Sumagin et al., 2010). Blocking or deletion of Mac-1 dramatically inhibits the infiltration of innate immune cells to the infected tissue and results in a significant defect in the clearance of the pathogens, indicating the importance of Mac-1 in innate immunity (Jiang et al., 1998; Phillipson et al., 2006).

Use of fluorescent cells has greatly facilitated cell tracking in live cell imaging. The fluorescent cells have been attained by labeling the cells with an exogenous dye or fluorescent dye-conjugated antibodies, and by fluorescent protein expression from a transgene (Gebhardt et al., 2011; Lammermann et al., 2013). Specifically, the transgenic reporter mouse expressing a fluorescent protein in a cell-type specific manner has been an important tool in studying leukocyte behavior and localization in tissue using various imaging strategies such as intravital two-photon microscopy. In this study, we generated a knock-in (KI) mouse by inserting the monomeric yellow fluorescent protein (mYFP) gene at the end of the final exon of CD11b gene, which resulted in the KI mouse expressing the CD11b protein conjugated with mYFP within its carboxyl terminus.

2. Materials and methods

2.1. Mice

The CD11b-mYFP KI mouse strain was generated at the Gene Targeting and Transgenic Core facility of the University of Rochester (Rochester, NY, USA). The KI mouse was back-crossed to C57BL/6 for more than 10 generations. C57BL/6 mice were purchased from the National Cancer Institute (NCI), and all of the mice in this study were maintained in a pathogen-free environment within the University of Rochester animal facility. The animal experiments were performed in compliance with the protocols approved by the University Committee on Animal Resources at the University of Rochester.

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2.2. Genotyping

The forward PCR primer was designed to anneal to the end of the last exon of the CD11b gene and the reverse primer to the region corresponding to 3′ untranslated part of the CD11b mRNA (forward primer 5′-AGTACAAGGACATGATGAATGAAGCT-3′ and reverse primer 5′-TGAGCACCTAAACCCTTGCAA-3′). PCR with this pair of primers amplifies 522 bp- and 1242 bp-long DNA fragments from WT and CD11b-mYFP KI alleles, respectively.

2.3. Epifluorescent microscopy

Fluorescence from splenocytes or neutrophils on a coverslip coated with 10 μg/ml recombinant mouse ICAM-1 (Sino Biological) or fibrinogen was captured using Nikon TE2000-E microscope. Neutrophils were purified from mouse bone marrow using an EasySep neutrophil enrichment kit (STEMCELL Technologies). To induce adhesion and migration, neutrophils were incubated with 2 μM fMLP (Sigma) at 37 °C during imaging.

2.4. Adhesion, phagocytosis, and transmigration assays

For adhesion assays, neutrophils were placed on an ICAM-1- or fibrinogen-coated glass slide with or without fMLP at 37 °C. Unbound cells were removed 15 min later and the number of bound cells was counted using microscopy. For phagocytosis assays, 100 μl of heparinized mouse blood was incubated with 100 μl of reconstituted pHrodo Red *Staphylococcus aureus* Bioparticles (Invitrogen) at 37 °C or 4 °C. After incubation for the indicated time, the red blood cells were lysed and the remaining white blood cells were harvested. Using fluorescence

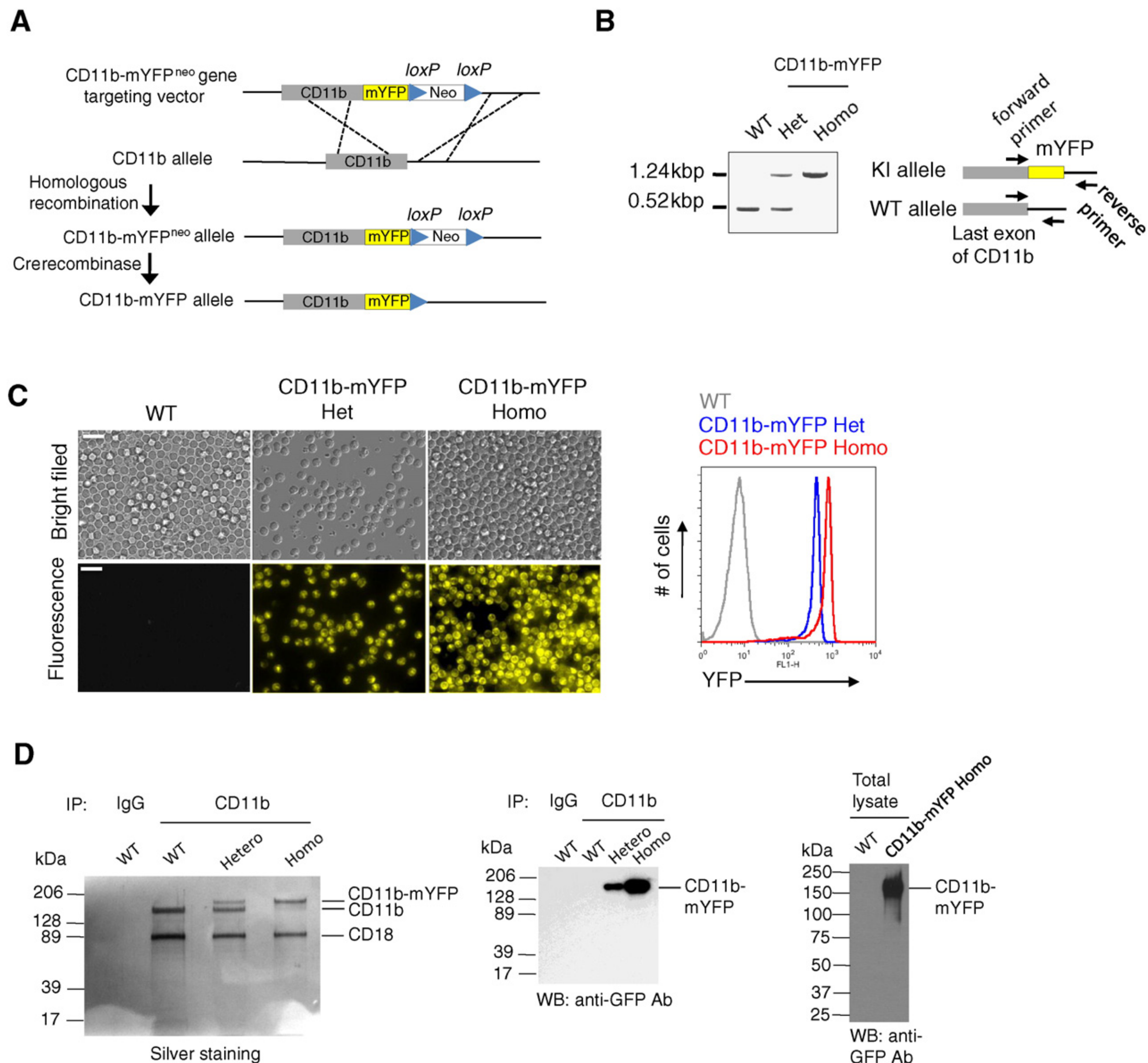

Fig. 1. Generation of CD11b-mYFP knock-in mouse. (A) Scheme for generation of CD11b-mYFP knock-in (KI) mouse strain by homologous recombination and loxP/Cre recombinase system. Neo, neomycin-resistant gene. (B) Selection of the CD11b-mYFP mouse by genotyping. PCR with primers flanking mYFP insertion site (arrows on the right) amplified distinct DNA fragments from CD11b alleles of the indicated mouse strains. Het, CD11b-mYFP heterozygous; Homo, CD11b-mYFP homozygous. (C) YFP fluorescence from CD11b-mYFP KI mouse strains. Fluorescence signals of neutrophils from WT, heterozygous or homozygous CD11b-mYFP KI mouse were detected using epifluorescent microscopy (left panels) or flow cytometry (right graph). Scale bar, 20 μm. (D) Integrity of CD11b-mYFP molecule. CD11b proteins were purified by immunoprecipitating total lysates of splenocytes from the indicated mouse strains using an anti-mouse CD11b monoclonal antibody (M1/70) or its isotype control antibody, rat IgG2b (IgG). The immunoprecipitates or the total lysates were analyzed using silver staining (left) or Western blotting (center and right) using the indicated antibodies. (E) Specific cell types expressing CD11b-mYFP. Splenocytes from CD11b-mYFP KI mouse were co-stained with anti-CD45.2 and −Ly6G, −F4/80, −Gr1, or −CD11c antibodies, and analyzed by flow cytometry. The graphs on the left show YFP levels *versus* cell-specific surface markers of the CD45.2-positive populations. Numbers indicate the percentage of gated cell populations which are YFP-positive and the indicated surface marker-positive. The graph on the right shows the percentage of YFP- and the indicated surface marker-double positive cells from splenocytes (mean ± SEM, n = 3).

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