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Dock5 controls the peripheral B cell differentiation via regulating BCR signaling and actin reorganization

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ABSTRACT

As an atypical guanine nucleotide exchange factor (GEF), Dock5 has been extensively studied in cellular functions. However, the role of Dock5 on B-cell immunity still remain elusive. In this study, we generated a Dock5 knockout mouse model to study the effect of Dock5 deficiency on B cell development, differentiation and BCR signaling. We found that the absence of Dock5 leads to a moderate effect on B cell development in the bone marrow and reduces follicular (FO) and marginal zone (MZ) B cells. Mechanistically, the key positive upstream B-cell receptor (BCR) signaling molecules, CD19 and Brutons tyrosine kinase (Btk), whose activation determines the fate of FO and MZ B cells, is reduced in Dock5 KO B cells upon antigenic stimulation by using total internal reflection fluorscence microscopy (TIRF) and immunoblot. Interestingly we found that the cellular filamentous actin (F-actin), also decreased in Dock5 KO B cells upon stimulation, which, in turn, offers feedback to BCR signaling. Our study has unveiled that Dock5 regulates the peripheral B cell differentiation via controlling the CD19-Btk signaling axis as well as actin reorganization.

1. Introduction

Dock (dedicator of cytokinesis) proteins, known as the activator to Rho family GTPases, are thought to regulate development, autoimmunity and bone homeostasis [1]. Dock5, a protein weighing 180KD, which belongs to the Dock-A subfamily (Dock1, Dock2 and Dock5) and functions as activators of small G protein Rac, is involved in intracellular signalling networks. Dock2, mainly expressed in hematopoietic cells, plays a role in regulating migration of leukocytes and activation of T cells. Moreover, it may also be a regulatory target for immune-related disorders. Dock2 and Dock5 can coregulate the formation of neutrophil extracellular traps (NETs) which involved in vascular inflammation and autoimmune responses [2,3]. Dock8, a member of another Dock subfamily, cause immunodeficiency syndromes in humans [3]. As one of Dock proteins, Dock5 now has been clearly identified to regulate multiple cellular functions, including myoblast fusion, bone resorption, migration, epithelial function and the rupture of murine lens cataracts [4–6]. But its role in immune system and immune response are still poorly understood.

B-cell receptor (BCR) signaling is indispensable for the development, survival and function of bone marrow and peripheral B cells. Antigen binding with BCRs induces the aggregation of BCR into microclusters and tranduces BCR signaling into cells, which subsequently activates the downstream signaling, such as Lyn, Syk, PLCγ2 and Brutons tyrosine kinase (Btk) [7,8]. These molecules allow BCR microclusters to grow over time and gradually fuse with each other, promoting the formation of BCR central clusters at one pole of the cell [9,10]. After the initial activation, the negative signaling molecules, such as SHIP, PTEN, are activated to decrease the signaling of the BCRs [11,12].

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Marginal zone (MZ) B cells, represented as a distinct population of presumably naive B cells, reside in close to the spleen marginal sinus region where resident immune cells are exposed to the open blood circulation [13], and play a pivotal role in defending against blood-borne encapsulated bacteria [14]. Upon antigen recognition, MZ B cells can rapidly proliferate and differentiate into plasma cells largely independent of T cell help. These plasma cells produce low-affinity antibodies to establish the first line of defense against the pathogens, leaving time for Follicular (FO) B cells to cooperate with T cells to generate the high-affinity antibody response [15,16]. It is believed that MZ B cells derived from FO B cells and the generation and maintenance of MZ B cells are markedly impaired in the absence of CD19 [17]. So far, no research has indicated the role of Dock5 on the differentiation of peripheral B cells.

Total internal reflection fluorescence microscopy (TIRFm) is an advanced imaging system to characterize the early events of B-cell activation stimulated by mAgs in vitro. It is based on the model system of antigen presented on lipid bilayers to mimic mAgs in vivo [18]. The role of actin on BCR signaling has been well characterized with TIRFm coupled with single-molecule tracking techniques. Actin is known to involved in the movement of BCRs. Actin regulates BCR lateral movement and aggregation by the way of polymerization and depolymerization, and that is essential for the morphological changes of B cells [19].

In this study, we have generated Dock5-deficient mouse model to examine the cellular function of Dock5 during BCR activation. We found that absence of Dock5 leads to downregulation of BCR signaling through decreased activation of CD19 and Btk, which is essential for the differentiation of FO and MZ B cells. Intriguingly, actin accumulation is also reduced in Dock5 B cells upon antigenic stimulation, which offers feedback to BCR signaling according to our previous research. Overall, our study has established a novel role of Dock5 in peripheral B cell differentiation as well as its underlying mechanism.

2. Materials and methods

All experiments involving mouse samples were performed using protocols approved by Chongqing Medical University animal care and usage committee and following institutional and NIH guidelines and regulations.

2.1. Mice and cells

Wild-type(wt) mice, on a C57 background, were purchased from animal center of Chongqing Medical University. Dock5 knockout (Dock5$^{-/-}$) mice were generously provided by Dr. Cote and Vuori of the Burnham Institute for Medical Research, La Jolla, CA. Splenic B cells were isolated as previously reported [20].

2.2. Preparation of Antigen-tethered planar lipid bilayers

The planar lipid bilayer was prepared as described previously [21]. Liposome were made by sonicating 1,2-dioleoyl-*sn-Glycero*-3-phosphocholine and 1,2-dioleoyl- *Glycero*-3-phosphoethanolamine-cap-biotin (Avanti Polar Lipids, Alabaster, AL) in a 100:1 M ratio in sterile PBS. Aggregates in liposomes were discarded by ultra centrifugation and filtration. Liposomes were incubated in coverslip chambers (Nalge Nunc International, Rochester, NY) for 20 min before coating with 1 ug/ml streptavidin (Jackson ImmunoResearch) followed by 5 ug/ml mB-Fab'-anti-Ig antibody.

2.3. Total internal reflection fluorescence microscopy

Images were acquired using a Nikon A1R confocal and TIRF system on an inverted microscope (Nikon Eclipse Ti-PFS), equipped with a 60X, NA 1.49 Apochromat TIRF objective (Nikon Instruments), an iXon EM-CCD camera (Andor), and 2 solid-state lasers with wavelengths 488 and 546 nm.

To image intracellular-signaling molecules, B cells were incubated with AF546-mB -Fab'-anti-Ig, tethered lipid bilayers at 37 °C for different time points, and then were fixed with 4% paraformaldehyde, permeabilized with 0.05% saponin, and last stained for phosphorylated Btk (pBtk, Y551;BD Bioscience), phosphorylated CD19 (pCD19, Y531; Abcam) and actin (AF488-phalloidin). The B-cell contact area and MFI of Fab'-anti-Ig in the B cell contact zone were determined using IRM images and NIS-Elements AR 3.2 software. Background fluorescence generated by Ag tethered to lipid bilayers in the absence of B cells was subtracted. For each set of data, > 20 individual cells were analyzed.

2.4. Flow cytometry analysis

Cell suspensions from bone marrow and spleen were stained for surface antigens in PBS supplemented with 2% FBS for 30 min on ice by using the following mAbs purchased from Biolegend: phycoerythrin (PE) anti-Ly51 (108307), allophycocyanin (APC) anti-CD43 (143208), PerCP anti-B220 (103234), PE-Cy7 anti- CD24 (101822), Brilliant Violet 421 anti-IgM (406518), FITC anti-CD19 (115506), PE anti-CD23 (101608), PerCP anti-IgD (405710), APC anti-CD21 (123412). Stained cells were analyzed by a BD FACS Canto and analyzed using FlowJo software .

2.5. Western Blot

Lysates, generated from B cells, were analyzed by SDS-PAGE and Western Blotting. Blots were probed with pBtk-(Y551; BD Bioscience), pAkt-(Ser473; Cell Signaling Technology) and pCD19-(Y531; Abcam) specific antibodies, and GAPDH- specific antibody (BBI Life Science) as loading controls.

2.6. Statistical analysis

Statistical significance was assessed by the two-tailed student's *t* test using Prism software (GraphPad software, San Diego, CA).

3. Results

3.1. The loss of Dock5 leads to decreased frequency and numbers of FO and MZ B cells

The role of Dock5 in the development of B cells has not been clearly reported. First, we detect the development of bone marrow-derived B cells in the Dock5$^{-/-}$ mice by using flow cytometry. Cells were stained with BP-1 and CD24 antibodies to distinguish pre-pro, pro, early-pre subsets, and B220-IgM antibody to distinguish late-pre, immature and recirculating B cells. A decreased number and percentage of early pre-B cells and recirculating B cells were found in Dock5$^{-/-}$ B cells, and no distinct difference was found in other B-cell subsets (Fig. 1A-D). This suggests that the development of bone marrow B cells were slightly affected in Dock5$^{-/-}$ mice. To further detect how Dock5 affects the peripheral B cells, IgM-IgD antibodies were used to distinguish the T1, T2 and FO B cells, CD23-CD21 antibodies to indicate the MZ B cells. The percentage and number of T1 and T2 B cells did not have any changes, but FO B cells were decreased in Dock5$^{-/-}$ B cells, and a further decrease was observed in MZ B cells (Fig. 1E-I). All these results suggest that Dock5 is indispensable for peripheral B cells.

3.2. The absence of Dock5 results in the diminished BCR signaling

It is reported that the generation of MZ B cells is reduced in the absence of CD19 mediated Btk signaling axis[22], thus we analyzed the expression levels of pCD19 and pBtk in Dock5$^{-/-}$-B cells upon sAg stimulation using immunoblot. We found that the phosphorylation

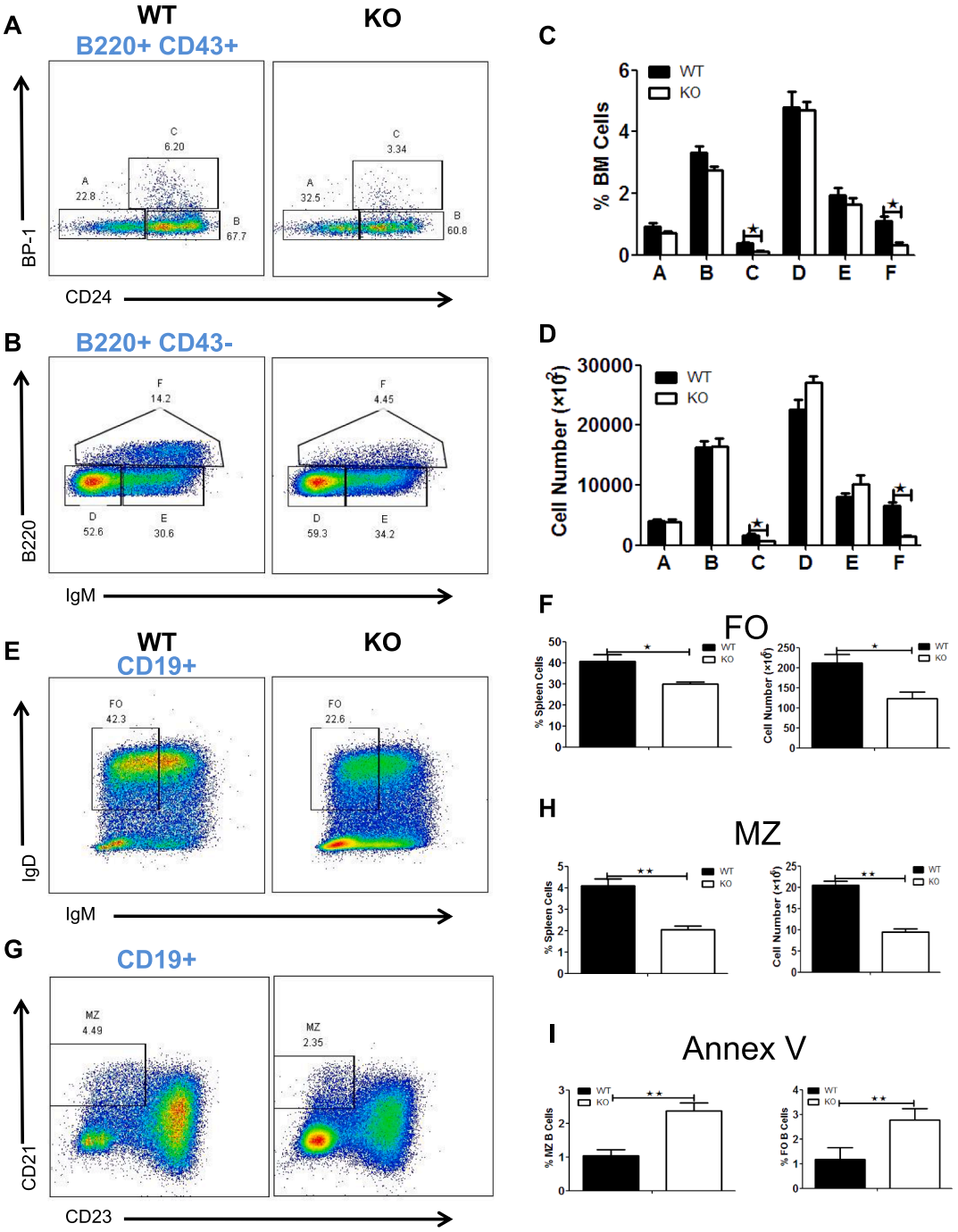

Fig. 1. Dock5 deficiency affects B-cell development in the Bone-marrow and reduces the differentiation of follicular and marginal-zone B cells. Flow cytometry analysis of Bone-marrow development. Gating strategy was as follows: A-pre-pro-B ($BP1^-CD24^-$), B-pro-B ($BP1^-CD24^+$) and C-early pre-B cells ($BP1^+CD24^+$) were gated from $B220^+CD43^+$ cells. D-late pre-B ($B220^+IgM^-$), E-immature B ($B220^{Int}IgM^+$) and F-recirculating B cells ($B220^{hi}IgM^+$) were gated from $B220^+CD43^-$ phenotype (A, C). The quantification of percentage and number of BM subsets in WT and $Dock5^{-/-}$ mice (B, D). Flow cytometry analysis of FO B ($CD19^+IgM^-IgD^+$) and MZ B cells ($CD19^+CD23^{lo}CD21^{hi}$) in the spleen (E, G). The quantification of percentage and number of FO and MZ B cells in the spleen of WT and $Dock5^{-/-}$ mice, respectively (F, H and I). The data shown are representative of WT (n = 5) and $Dock5^{-/-}$ (n = 5) mice.*$p < .01$; **$p < .001$.

levels of CD19 and Btk were reduced in Dock5 KO B cells compared to that of WT B cells upon sAg stimulation (Fig. 2A-D). B cells lacking CD19 signaling are defective in BCR clustering and B-cell spreading, resulting in a series of downstream signaling defects, such as PI3K, Btk and Akt. We examined the activation levels of distal BCR signaling molecule-Akt. We found that the activation of Akt was also impaired in Dock5 KO B cells upon stimulation (Fig. 2E,F). Altogether these results suggest that Dock5 positively regulates the proximal and distal BCR signaling.

3.3. The absence of Dock5 leads to the reduction of the recruitment of BCR signalosome in the contact zone of $Dock5^{-/-}$ B cells upon mAg stimulation

To further study the mechanism of Dock5 deficiency on the BCR signaling, we used TIRFm to examine the early activation of Dock5 KO B cells. The decreased BCR signaling in Dock5 KO B cells upon sAg stimulation implies disruption in early B cell activation. To test this hypothesis, we analyzed the impact of Dock5 deficiency on the BCR clustering and BCR signalosome recruitment at the cell surface in

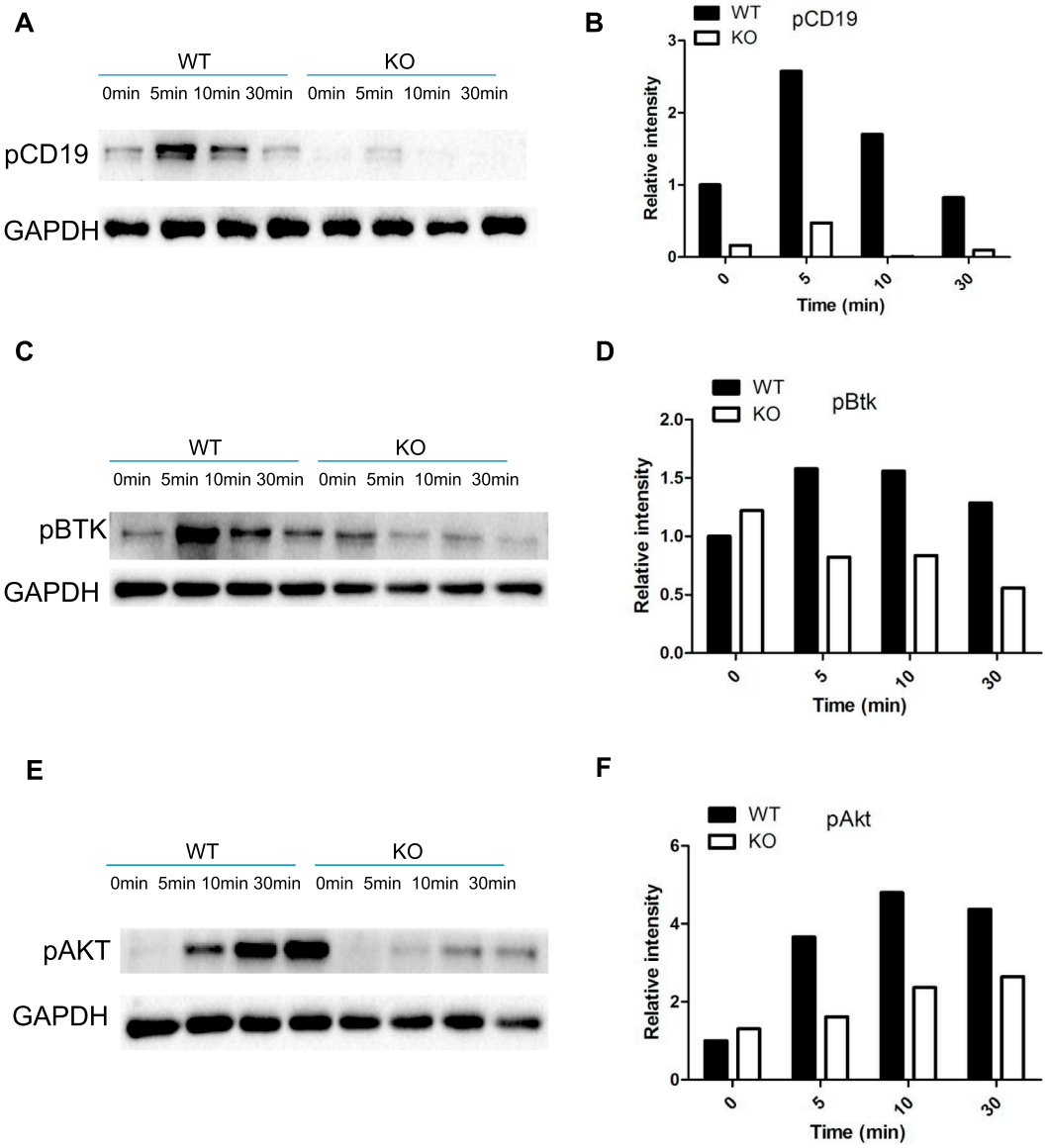

Fig. 2. The levels of pCD19, pBtk and pAkt in B cell receptor (BCR) clusters are reduced in Dock5 knockout (KO) B cells. Western Blot analysis of the pCD19, pBtk and pAkt after stimulation with sAg at different time points (A, C and E); GAPDH was used as a loading control. Shown are representative immunoblots at different times (B, D and F).

response to membrane-tethered Fab'-anti-Ig using TIRFm. In WT B cells, the contact area increased for the first 5 min and followed to contract. The contact area dynamics of Dock5 KO B cells was similar to that of WT B cells, but the contact area size was drastically decreased (Fig. 3E). The mean fluorescence intensity (MFI) of the BCR cluster increased over time in both WT and KO B cells, but the intensity in KO B cells was much less than WT B cells (Fig. 3F). The MFI of pCD19 in the contact zone of WT B cells rapidly increased upon antigen binding, reached a peak at 5 min, and then decreased. The MFI of pCD19 in KO B cells shared a similar distribution and level as in WT B cells, but the increasing magnitude of pCD19 MFI was significantly lower than that of WT B cells (Fig. 3A,B,G). Similar to the pCD19, the MFI of pBtk also peaked at 5 min and was significantly reduced in the contact zone of Dock5 KO B cells compared to that of WT B cell (Fig. 3C,D,H). These results indicate that Dock5 is critical for the formation of BCR clustering and essential for the consequential BCR signaling.

3.4. Dock5 inhibits F-actin accumulation in the B-cell contact zone

Since actin reorganization is closely related to B-cell spreading and contraction, we used the TIRFm system to examine the levels of F-actin stained with phalloidin in the B-cell contact zone. The level of F-actin in the contact zone of control B cells increased over time and reached a peak at 3 min accompanied by B-cell stretching to its maximal magnitude, then the B-cell began to contract followed by a reduction at 7 min. Dock5 deficiency did not change this distribution pattern of F-actin but had a much lower level of F-actin accumulation in the B-cell contact zone (Fig. 4A-C). These results imply that Dock5 positively regulates the actin reorganization.

4. Discussion

In this study, we investigated the role of Dock5$^{-/-}$ on BCR signaling by using germline Dock5$^{-/-}$ mice. This present study provides a novel view of Dock5 being involved in the development of B cells and

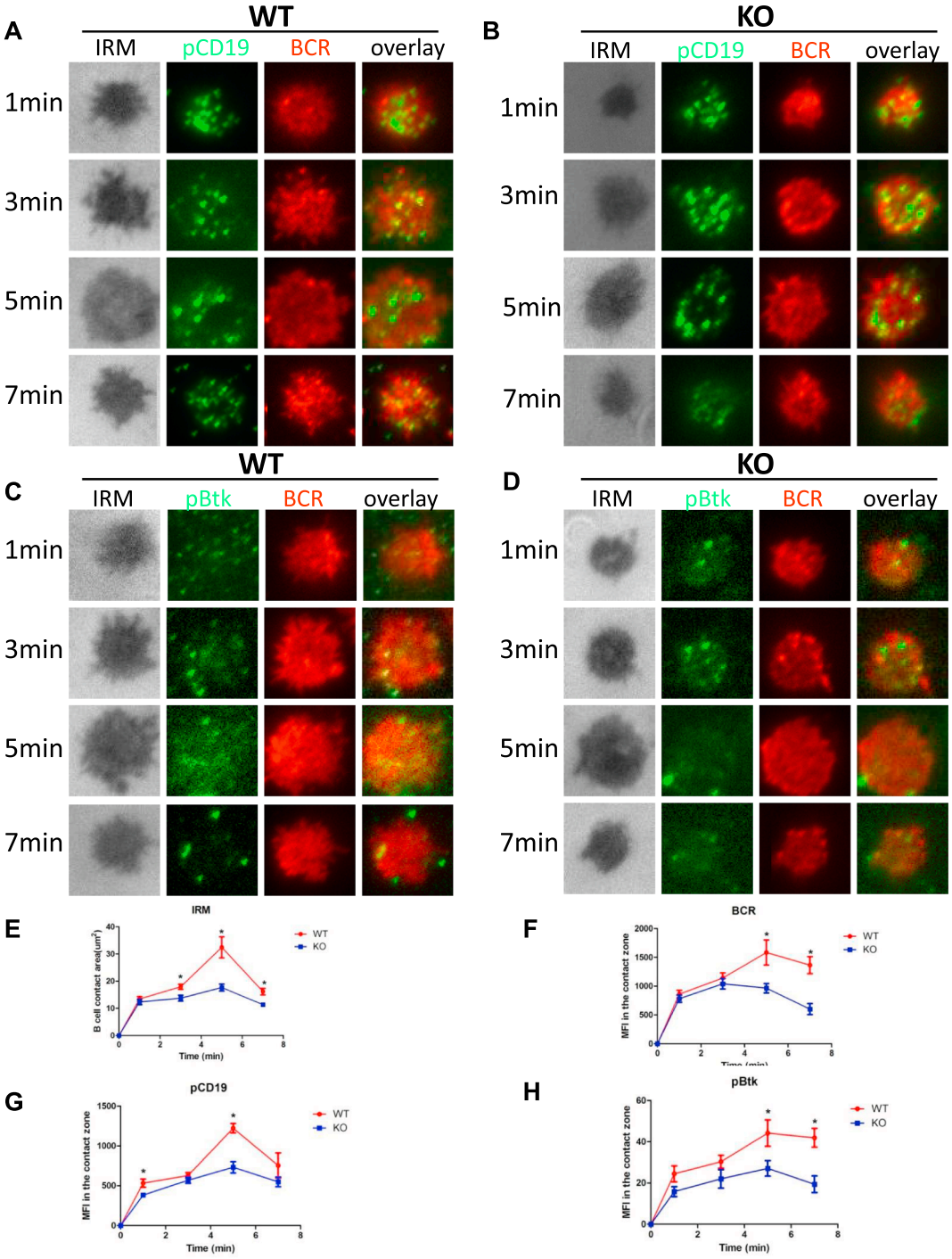

Fig. 3. Dock5 deficiency disrupts the early activation of B cells. TIRFm analysis of pCD19 and pBtk staining in the contact zone of splenic B cells from wild-type (WT) and Dock5 KO mice incubated with membrane-tethered Fab'-anti-Ig. Shown are representative images (A-D) and the B-cell contact area (E), the MFI of BCR in the B-cell contact zone (F), MFI of pCD19 (G) and pBtk (H) in the B-cell contact zone. Scale bars, 2.5 μm.*P < .01.

BCR signaling. This is shown by slightly reduced bone marrow B cells subsets, and decreased FO and MZ B cells in Dock5$^{-/-}$ mice peripheral B cells. Additionally, KO B cells had attenuated activation of the key positive BCR signaling molecules, Btk and the upstream molecule of Btk-CD19. Furthermore, F-actin intensity is reduced in Dock5$^{-/-}$ B cells. We need to get Dock5 flox mice to cross with CD19Cre mice to get Dock5 deletion in B cells specifically or bone marrow chimera mice to confirm the effect of Dock5 deficiency on the B cell development.

One interesting direction needs to be studied is how Dock5 up-regulates the B-cell signaling. There are currently 11 known Dock proteins [23], Dock8 has been well investigated in immune deficiency. Dock2 is involved in the activation of lymphocytes [3]. This promotes the question of whether Dock5 play the similar role in humoral immunity like Dock2 or Dock8. A previous study showed that Dock8 play a putative role in actin cytoskeleton reorganization through Rho GTPases, such as Cdc42 and Rac1. These GTPases, in turn, are regulated by the activity of Dock8 [23]. Recently, Dock8 was reported to regulate BCR signaling through regulatingWASP [24], which acts as a downstream effector for signals from Rho GTPases. Similarly the Dock8-WIP-WASP axis was also reported to link the TCR with actin [25]. Based on

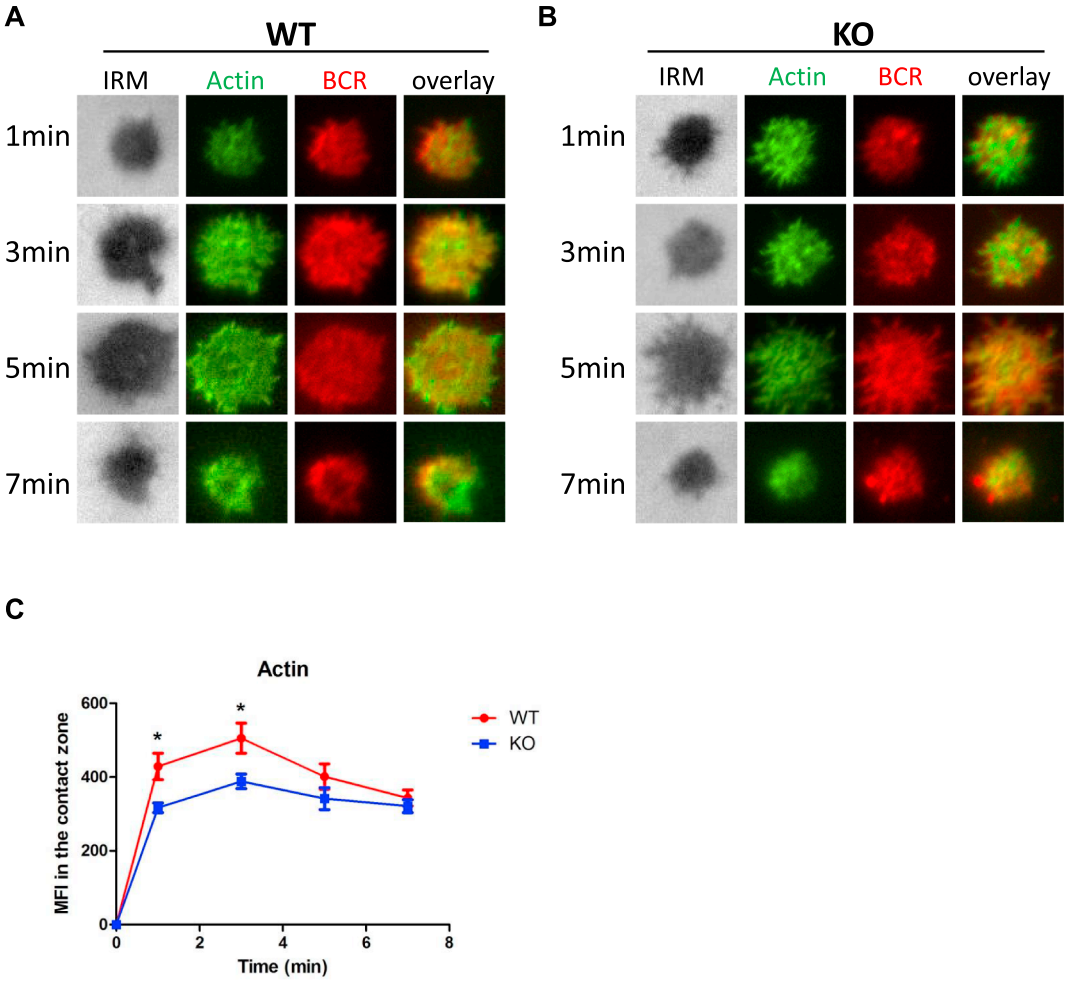

Fig. 4. Dock5 promotes F-actin accumulation in the B-cell contact zone. TIRFm analysis of F-actin staining in the contact zone of mouse splenic B cells from wild-type (WT) and Dock KO mice that were incubated with membrane-tethered Fab'-anti-Ig. Shown are representative images (A,B) and the MFI of F-actin in the B-cell contact zone (C). Scale bars, 2.5 μm.*P < .01.

these findings and our results, we provide a hypothesis that Dock5 may regulate the BCR signaling through WAS and WIP. Therefore we are going to examine the levels of WASP and WIP in Dock5 KO B cells upon stimulation.

The other interesting future direction to be determined is how Dock5 regulate F-actin. In our studies, we found a decreased level of F-actin accumulation in the B-cell contact zone of Dock5 KO B cells. Our previous studies have shown that actin can offer positive and negative feedback to BCR signaling, such as WASP stimulating actin polymerization by binding Arp2/3 to modulate BCR lateral mobility and drive B-cell spreading, while N-WASP can reduce the surface level of F-actin probably by depolymerizing [26]. Rictor can lead to decreased actin polymerization via ezrin and Dock8 can enhance polymerization of actin via WASp [24,27]. These results indicate Dock5 may affect one of these to regulate actin polymerization. All together, we further need to detect the signaling of downstream molecules, such as WASP and Ezrin to define the detail underlying mechanism during Dock5$^{-/-}$ B cell activation.

In summary, our study initially reveals a new phenomenon that Dock5 can regulate BCR signaling. Dock5 deficiency leads to dysregulation of phosphorylation of CD19 and Btk, induces dysregulation of actin accumulation, and ultimately impact B-cell differentiation. The dissection of the underlying mechanism of how Dock5 regulates BCR signaling and B cell differentiation can provide therapeutic designs to the primary immunodeficiency disease caused by Dock5 mutation.

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Conflicts of Interest

The authors have no financial conflict of interest.

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