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Critical role of integrin CD11c in splenic dendritic cell capture of missing-self CD47 cells to induce adaptive immunity

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CD11c, also known as integrin alpha X, is the most widely used defining marker for dendritic cells (DCs). CD11c can bind complement iC3b and mediate phagocytosis in vitro, for which it is also referred to as complement receptor 4. However, the functions of this prominent marker protein in DCs, especially in vivo, remain poorly defined. Here, in the process of studying DC activation and immune responses induced by cells lacking self-CD47, we found that DC capture of CD47-deficient cells and DC activation was dependent on the integrin-signaling adaptor Talin1. Specifically, CD11c and its partner Itgb2 were required for DC capture of CD47-deficient cells. CD11b was not necessary for this process but could partially compensate in the absence of CD11c. Mice with DCs lacking Talin1, Itgb2, or CD11c were defective in supporting T-cell proliferation and differentiation induced by CD47-deficient cell associated antigen. These findings establish a critical role for CD11c in DC antigen uptake and activation in vivo. They may also contribute to understanding the functional mechanism of CD47-blockade therapies.

CD11c | CD47 | integrin | talin | dendritic cell

Dendritic cells (DCs) play critical roles in capturing and presenting antigens to T cells, a process that is essential for shaping host immune responses against infection and cancer (1). In the spleen, classical DCs (cDCs) can be divided into two subsets based on their dependence on lineage-specific transcriptional factors and surface marker expression: cDC1s develop in a Batf-3– and IRF8-dependent manner and express CD8α and XCR1, whereas cDC2s require IRF4 for their development and are marked by the expression of CD4, CD11b, DCIR2, and SIRPα (1, 2). Splenic cDC2s strategically localize in the marginal zone (MZ) bridging channel, a unique region in the spleen that extends between blood-exposed red pulp and lymphocyte-rich white pulp (3–5). Upon exposure to blood-borne antigen, cDC2s become activated and move to the T-cell zone to prime $CD4^+$ T cells. This translocation event is controlled by the chemokine receptor CCR7 (3, 6, 7).

Known as "marker of self," CD47 is a cell surface protein abundantly expressed by most cell types. CD47 engagement of the highly polymorphic signal regulatory protein α (SIRPα) receptor on phagocytes transmits a "don't eat me" signal that prevents cell engulfment (8). CD47 is up-regulated in multiple human tumor types such as leukemia and lymphoma, allowing tumor cells to evade ingestion by phagocytes (9, 10). In preclinical models using immunodeficient mice, CD47 antagonism-based therapies have led to the reduction of xenografted human tumors by promoting macrophage-dependent clearance (10, 11). However, very recently, it has become increasingly appreciated that the therapeutic efficacy of CD47 blockade in syngeneic tumor models requires DCs to activate adaptive immune responses (12, 13).

Xenogeneic red blood cells (RBCs), such as sheep RBCs (SRBCs), have been widely used as a model antigen for triggering systemic immune responses, yet the basis for their adjuvant activity had been unknown. Our previous effort in understanding the mechanism of SRBC immunogenicity led to the finding that sheep CD47 fails to bind mouse SIRPα, and therefore SRBCs can be captured and cause splenic cDC2 activation to elicit immune responses (14). The mechanism of DC uptake of CD47-deficient cells and DC activation was found to involve integrin β2 (Itgb2) but was otherwise not understood (14).

Integrins are α/β heterodimeric cell surface receptors that are involved in a wide range of adhesion-related cellular processes (15). The activation of integrins is controlled by Talin1 and Talin2, adaptor proteins important for switching integrins to conformations with high affinity for their ligands (16). DCs highly express Talin1 while lacking expression of Talin2 (ref. 14; Immgen.org). CD11c, also known as integrin αX (gene name *Itgax*), is the most widely used defining marker for DCs. CD11c pairs with Itgb2, and this integrin heterodimer has been shown to bind complement fragment iC3b and mediate phagocytosis in vitro, for which it is also referred to as complement receptor 4 (17, 18). It has also been found to interact with other soluble molecules, such as heparin and fibrinogen, and with adhesion molecules such as ICAM-1 (19, 20). However, the function of this prominent marker protein in DCs, especially in vivo, remains poorly defined.

Significance

The cell surface protein CD47 functions as a self-recognition molecule on mammalian cells, sending a negative "don't eat me" signal to macrophages and dendritic cells. When CD47 is missing from cells in circulation, they are promptly taken up by and cause activation of dendritic cells in the spleen, and this, in turn, leads to stimulation of the adaptive immune system. The positive recognition system used by dendritic cells to capture CD47-deficient cells has been unknown. Here we show that the integrin CD11c, a classical marker of dendritic cells, and cytoskeletal protein talin are critically involved in binding and uptake of missing-self CD47 cells. These findings may advance efforts to use CD47 blockade therapies for treatment of cancer.

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Conflict of interest statement: J.G.C. is on the Scientific Advisory Board of Alexo Therapeutics Inc., a company developing CD47 targeted therapeutics.

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In this study, we have dissected the role of integrins in DC responses to cells lacking self-CD47. We found that several types of cells from CD47-deficient mice could cause splenic DCIR2$^+$ cDC2 activation. DC capture of CD47-deficient cells and DC activation were dependent on the integrin-signaling adaptor Talin1. After immunization with SRBCs, Talin1-deficient cDC2s failed to support follicular helper T (Tfh) cell differentiation, as well as T-dependent B-cell responses. Examination of integrin α chain requirement for DC capture of CD47-deficient cells revealed an unexpected dependence on CD11c. CD11b was not essential but could be redundantly involved when CD11c was removed. Mice with DCs lacking CD11c were defective in supporting T-cell proliferation and differentiation induced by CD47-deficient cell associated antigen.

Results

Capture of CD47-Deficient Cells by DCIR2$^+$ Splenic DCs. To identify cDC2s in the spleen, we took advantage of their unique expression of the C-type lectin receptor DCIR2 (3, 5). Distinct from cDC1s, DCIR2$^+$ cDC2s express a high level of inhibitory receptor SIRPα. We have previously shown that sheep CD47 fails to engage mouse SIRPα and this causes SRBCs to activate splenic DCIR2$^+$ cDC2s to induce adaptive immune responses (14). Introduction of red fluorescent dye PKH26 labeled-SRBCs into mouse circulation led to labeling of a large fraction of splenic DCIR2$^+$ DCs (*SI Appendix*, Fig. S1*A*). Consistently, RBCs from *Cd47*$^{-/-}$ but not *Cd47*$^{+/-}$ mice, when transfused into WT recipients, were readily captured by splenic DCIR2$^+$ DCs and caused their up-regulation of activation markers CCR7, CD86, and MHC-II (*SI Appendix*, Fig. S1 *B* and *C*). The DC-stimulatory activity of CD47-deficent cells was not unique to RBCs. When freshly isolated *Cd47*$^{-/-}$ thymocytes and bone marrow (BM) cells were RBC-depleted and then injected into WT mice, similar capture and DCIR2$^+$ DC activation was observed (*SI Appendix*, Fig. S1 *B* and *D*).

Talin1 Is Required for DC Capture and DC Activation by CD47-Deficient Cells. We next sought to understand the signaling mechanisms of DC capture of *Cd47*$^{-/-}$ cells and DC activation. We have previously established an involvement of Src family tyrosine kinases (SFKs) and the integrin β2 chain in *Cd47*$^{-/-}$ RBC-induced DCIR2$^+$ DC activation (14). Integrins are one of many classes of transmembrane receptors that can activate SFKs (21). To broadly test the need for integrin activity in CD47-deficient cell-induced DC activation, we focused on Talin1, a key regulator of integrin signaling (16). We crossed a mouse line carrying floxed *Talin1* alleles (*Tln1*$^{fl/fl}$) (22) to mice expressing Cre recombinase under the control of the CD11c promoter (CD11c-Cre). CD11c-Cre caused efficient depletion of Talin protein in both cDC1s and cDC2s, whereas Talin expression in other cells was either less affected (plasmacytoid DCs) or unaffected (B cells) (*SI Appendix*, Fig. S2*A*). Talin1-deficient DCIR2$^+$ DCs were strongly compromised in their ability to capture *Cd47*$^{-/-}$ RBCs, thymocytes, and BM cells (Fig. 1*A*). They also showed minimal up-regulation of activation markers 3 h after stimulation by *Cd47*$^{-/-}$ RBCs or SRBCs (Fig. 1*B*). This defect was specific to CD47-deficient cell-induced activation, as cDC2 activation by other stimuli such as lipopolysaccharides (LPS) or heat-killed *Listeria monocytogenes* (HKLM) remained intact (Fig. 1*B* and *SI Appendix*, Fig. S2*B*).

After activation by various immune stimuli, WT DCIR2$^+$ DCs migrate from the MZ bridging channels to the T-cell zone (Fig. 1*C*), a process dependent on the chemoattractant receptors CCR7 and EBI2 (3, 6, 23). In accord with their selective defect in

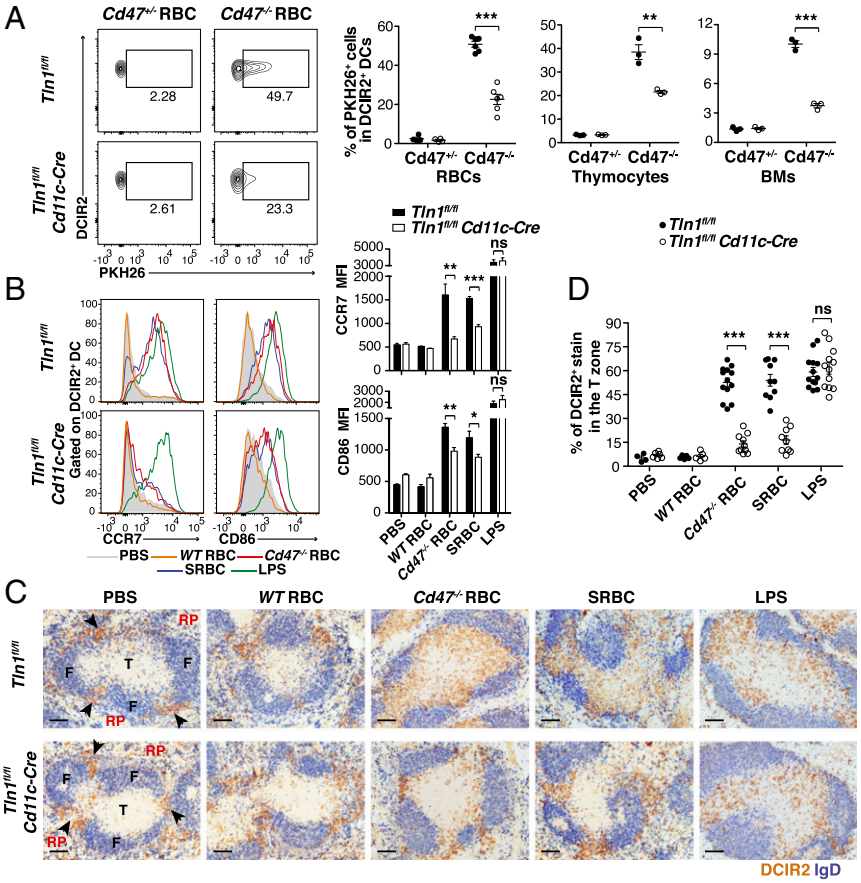

Fig. 1. Talin1 is required for splenic cDC2 capture of CD47-deficient cells and cDC2 activation. (*A*) Mice of indicated genotypes were i.v.-injected with the indicated type of PKH26-labeled cells from *Cd47*$^{+/-}$ or *Cd47*$^{-/-}$ donors and analyzed 3 h later by flow cytometry. Representative FACS plots (*Left*) and summary data (*Right*) of PKH26 acquisition in gated DCIR2$^+$ DCs are shown. (*B*) Representative FACS plots (*Left*) and summary data (*Right*; $n = 3$ to 5) for surface expression of CCR7 and CD86 in gated DCIR2$^+$ DCs from mice of specified genotypes 3 h after immunization with indicated stimuli. MFI, geometric mean fluorescence intensity. (*C*) Representative images showing the localization of splenic DCIR2$^+$ DCs (brown) relative to B cells (IgD$^+$, blue) in mice of specified genotypes 6 h after i.v. immunization with the indicated stimuli. Arrowheads in left-most panels point to the location of MZ bridging channels. F, follicle; RP, red pulp; T, T-cell zone. (Scale bars, 100 μm.) (*D*) Summary data for *C*; each point represents a white pulp cord. All data are representative of at least two independent experiments with three mice per group. Statistical analyses were performed using the unpaired two-tailed Student's *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant).

CCR7 induction by cells lacking self-CD47, Talin1-deficient DCIR2$^+$ DCs failed to relocate to the T-cell zone shortly after *Cd47*$^{-/-}$ RBC or SRBC immunization (Fig. 1 *C* and *D*). In contrast, relocation to the T-cell zone remained intact after LPS stimulation (Fig. 1 *C* and *D*), ruling out the involvement of Talin1-mediated integrin signaling in controlling general DC migration (24).

DC Talin1 Is Required for Priming T- and B-Cell Responses to RBCs Lacking Self-CD47. We next questioned whether the defective CD47-deficient cell capture and subsequent T-zone migration would impact the ability of Talin1-deficient DCIR2$^+$ DCs to prime T cells. We focused on Tfh cell induction because of the established role of cDC2 in development of these effector T cells (25). Control or DC Talin1-deficient mice were immunized with SRBCs, and, 6 d later, Tfh cell differentiation was examined. Talin1 deficiency in DCs resulted in a marked reduction in CXCR5$^+$PD-1^{high} Tfh cells (*SI Appendix*, Fig. S3*A*). CD11c-Cre was reported to be active in a small fraction of T cells (26). To fully rule out the potential contribution of T-cell–intrinsic Talin1 deletion in the observed Tfh cell defects, we adoptively transferred violet tracer-labeled WT ovalbumin (OVA)-specific OT-II T cells into control and DC Talin1-deficient mice. The recipient mice were then immunized with OVA-conjugated SRBCs. After 3 d, OT-II T cells in hosts that had Talin1-deficient DCs were strongly compromised in proliferation and up-regulation of Tfh cell markers CXCR5, PD-1, and ICOS (Fig. 2*A*). In contrast, after immunization by soluble OVA with LPS, comparable OT-II T-cell proliferation and differentiation was observed in control and DC Talin1-deficient hosts (Fig. 2*B*).

To test the effects on induction of T-dependent B-cell responses, DC Talin1-deficient or control mice were immunized with SRBCs, and, 6 d later, germinal center (GC) B-cell formation was assessed by GL7 and Fas up-regulation. Compared with their control littermates, mice lacking Talin1 in DCs formed drastically diminished GC compartments (Fig. 2*C*). In an adoptive transfer experiment using WT hen egg lysozyme (HEL)-specific Hy10 B cells, recipient mice with DC Talin1 deficiency were less able to support Hy10 B-cell proliferation and differentiation into GC B cells (Fig. 2*D*).

As well as exhibiting DC activation defects, DC Talin1-deficient mice were found to have reduced numbers of DCIR2$^+$ cDC2 (*SI Appendix*, Fig. S3*B*). To control for possible effects of the reduced DC number on T-cell activation, we reconstituted mice with BM from donors carrying diptheria toxin receptor fused to the cDC marker gene Zbtb46 (zDC-DTR) mixed with different ratios of control and DC Talin1-deficient BMs and then treated mice with Diphtheria Toxin. This BM chimeric approach generated control and DC Talin1-deficient mice with matched numbers of DCIR2$^+$ DCs (*SI Appendix*, Fig. S3*C*). Adoptive transfer experiments in these mice again showed a T-cell response defect selectively in mice lacking Talin1 in DC (*SI Appendix*, Fig. S3*D*). Together, these results establish an essential and specific role for DC intrinsic Talin1-dependent integrin signaling in supporting T-cell and T-dependent B-cell responses triggered by cells lacking self-CD47.

CD11c (αXβ2) Integrin Is Required for DC Capture of CD47-Deficient Cells to Prime T-Cell Responses. There are 18 α and 8 β integrin subunits, which combine to form at least 24 distinct α/β heterodimers (15). Our previous analysis showed an intrinsic role for the β2 integrin chain in *Cd47*$^{-/-}$ RBC uptake (14). In an RNAseq dataset of splenic DCs that we previously generated (GEO accession no. GSE71165), three additional β chain members (β1, β3, and β7) showed detectable expression in splenic DCIR2$^+$ DCs (*SI Appendix*, Fig. S4*A*). When BM chimera mice with DCs lacking individual β-chain family members were injected with *Cd47*$^{-/-}$ RBCs, DCIR2$^+$ DCs lacking β1, β3, or β7 integrins showed unaffected *Cd47*$^{-/-}$ RBC uptake compared with their littermate controls (*SI Appendix*, Fig. S4*B*), whereas DCs lacking β2 integrin almost completely lost the ability to

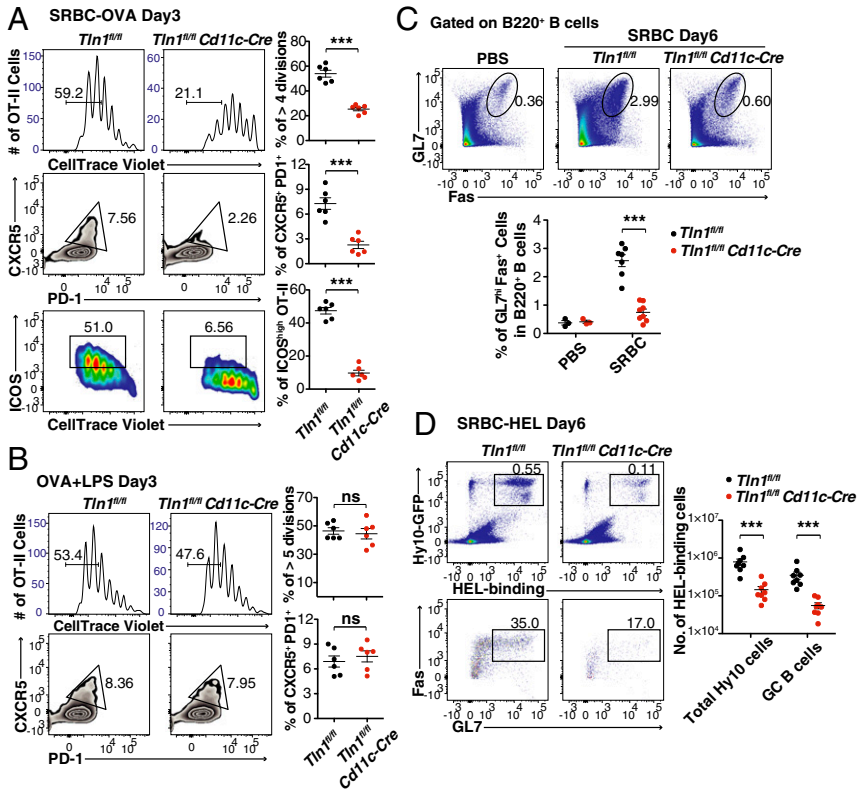

Fig. 2. Talin1-deficient cDC2s are incapable of supporting SRBC-induced Tfh cell and GC B-cell responses. (*A*) Representative FACS plots (*Left*) and summary data (*Right*) showing the proliferation measured by violet tracer dilution (*Upper*), up-regulation of CXCR5 and PD-1 (*Middle*), and up-regulation of ICOS (*Lower*) by transferred OT-II T cells in host mice of indicated genotypes at day 3 after SRBC-OVA immunization. Summary data are pooled from two experiments with three mice per group. (*B*) Representative FACS plots (*Left*) and summary data (*Right*) showing the proliferation measured by violet tracer dilution (*Upper*) and up-regulation of CXCR5 and PD-1 (*Lower*) by transferred OT-II T cells in host mice of indicated genotypes at day 3 after immunization with OVA and LPS. Summary data are pooled from two experiments with three mice per group. (*C*) Representative FACS plots (*Upper*) and summary data (*Lower*) of percentages of GL7^{high}Fas$^+$ GC B cells within gated B220$^+$ cells in mice of indicated genotype at day 6 after SRBC immunization. Summary data are pooled from two experiments with three to five mice per group. (*D*) Representative FACS plots of frequencies of transferred HEL-binding Hy10-GFP cells in total splenocytes (*Upper Left*) and percentages of GL7^{high}Fas$^+$ GC B cells within gated HEL-binding Hy10-GFP cells (*Lower Left*) from host mice of indicated genotypes at day 6 after SRBC-HEL immunization. Summary data (*Right*) showing the number of total and GL7^{high}Fas$^+$ HEL-binding Hy10-GFP cells are pooled from two experiments with three to five mice per group. Statistical analyses were performed using the unpaired two-tailed Student's *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant).

capture CD47-deficient RBCs and thymocytes (*SI Appendix*, Fig. S4*C*). Integrin β2-deficient DCs were also defective in up-regulating chemokine receptor CCR7 in response to $Cd47^{-/-}$ thymocyte stimulation (*SI Appendix*, Fig. S4*C*). The greater defect in $Cd47^{-/-}$ cell capture by β2-deficient DCs than Talin1-deficient DCs might reflect some compensatory role for other adaptor proteins in the Talin1-deficient cells (16).

The β2 integrin subunit can pair with four different α subunits, among which three (αL, αM, and αX, more commonly known as CD11a, CD11b, and CD11c) showed significant expression in the splenic $DCIR2^+$ DCs (*SI Appendix*, Fig. S5*A*). Blocking CD11a or CD11b subunits with neutralizing antibodies did not result in any defect in DC uptake of $Cd47^{-/-}$ RBCs (Fig. 3*A*). In contrast, a partial impairment in $Cd47^{-/-}$ RBC capture was observed after CD11c blockade (Fig. 3*A*). CD11c has been widely used as a defining marker for DCs. However, the exact function of this protein, especially in vivo, remains poorly defined. CD11c null ($Itgax^{-/-}$) mice showed normal frequencies of cDC2 and cDC1, as revealed by staining with unique markers of these two subsets, DCIR2 and XCR1 (*SI Appendix*, Fig. S5*B*). Consistent with the antibody blockade result, CD11c-deficient DCs were also impaired in capturing $Cd47^{-/-}$ RBCs and thymocytes (Fig. 3*B*). To test the role of DC CD11c in supporting CD47-deficient cell-induced T-cell responses, WT OT-II T cells were adoptively transferred into control or $Itgax^{-/-}$ BM full chimeras. Three days after immunization with SRBC-OVA, OT-II T cells in $Itgax^{-/-}$ hosts showed less proliferation and up-regulation of Tfh cell markers CXCR5, PD-1, and ICOS (Fig. 3*C*). Together, these findings established a role for CD11c integrin in missing-self CD47 cell capture and DC activation to prime Tfh cell responses.

CD11b Is Nonessential but Redundantly Involved in CD11c-Mediated DC Uptake of CD47-Deficent Cells to Support T-Cell Responses. The incomplete loss of $Cd47^{-/-}$ cell capture caused by CD11c deficiency compared with Itgb2 deficiency suggested that there might be redundancy between CD11c and other Itgb2-pairing integrins (Fig. 3*B* and *SI Appendix*, Fig. S4*B*). In this regard, CD11b is 66% identical (77% homologous) to CD11c over the β-propeller and ligand-binding I domains, and these integrins share a number of ligands, including ICAM1, iC3b, fibronectin, and fibrinogen (20). Indeed, while DCs lacking CD11b ($Itgam^{-/-}$) alone showed normal RBC uptake, blocking CD11c in $Itgam^{-/-}$ mice led to a more profound defect in $Cd47^{-/-}$ RBC capture (Fig. 4*A*). The partial redundancy of CD11c and CD11b was further confirmed by introducing CD11c and CD11b double deficiency with several combinations of antibody blockade and genetic deletions (Fig. 4*B*). These results suggest that, even though CD11b is not required by itself in CD47-deficient cell uptake, it can partially compensate when CD11c is missing.

In accord with the defects in CD47-deficient RBC capture, the proliferation and differentiation of adoptively transferred OT-II T cells was severely compromised when host mice had CD11c single deficiency and almost completely abrogated in hosts with CD11c and CD11b double deficiency (Figs. 3*C* and 4*C*). These results revealed a compensatory role for CD11b in CD11c-mediated capture of CD47-deficient cell-associated antigen and subsequent T-cell priming, which is otherwise indiscernible in mice with $DCIR2^+$ DCs lacking CD11b alone.

Discussion

The inhibitory "don't eat me" signal transduced by CD47–SIRPα interaction has been well characterized, but the activation signal that it counteracts has been unclear (8). Our finding that cells lacking self-CD47, and that fail to engage SIRPα, could induce rapid capture by splenic DCs provided a system to study the activation signals in vivo. Using this model, we have defined a role for CD11c and its regulator Talin1 in splenic cDC2 capture of cells lacking self-CD47 and subsequent DC activation in vivo. The inability of CD11c- or Talin1-deficient DCs to capture

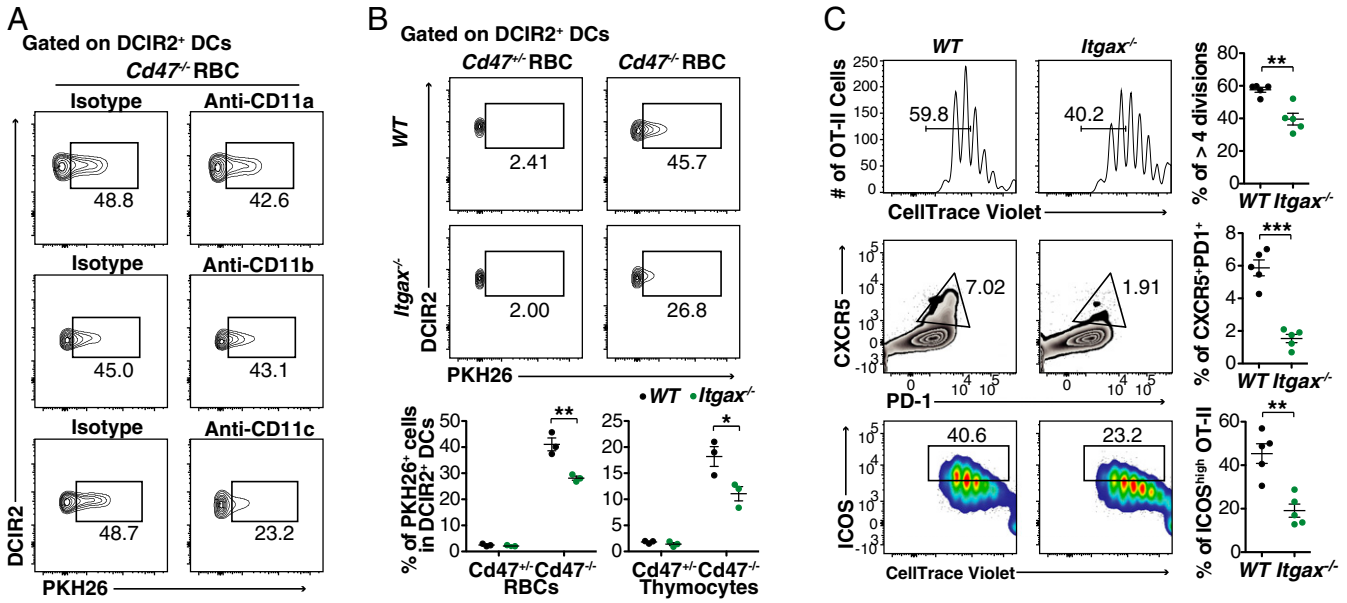

Fig. 3. Requirement of CD11c in $DCIR2^+$ DC capture of CD47-deficient cells to prime Tfh cell responses. (*A*) Representative FACS plots of PKH26 acquisition in gated $DCIR2^+$ DCs from mice pretreated with indicated neutralizing antibodies for 1 h followed by i.v. immunization with PKH26-labeled $Cd47^{-/-}$ RBCs for 3 h. (*B*) Mice of specified genotypes were i.v.-injected with indicated type of PKH26-labeled cells from $Cd47^{+/-}$ or $Cd47^{-/-}$ donors and analyzed 3 h later by flow cytometry. Representative FACS plots (*Upper*) and summary data (*Lower*) for PKH26 acquisition in gated $DCIR2^+$ DCs are shown. (*C*) Representative FACS plots and summary data showing the proliferation measured by violet tracer dilution (*Left*), up-regulation of CXCR5 and PD-1 (*Middle*), and up-regulation of ICOS (*Right*) by transferred OT-II T cells in BM chimera mice of indicated genotypes at day 3 after immunization with SRBC-OVA. Summary data are pooled from two experiments with two to three mice per group. Statistical analyses were performed using the unpaired two-tailed Student's *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

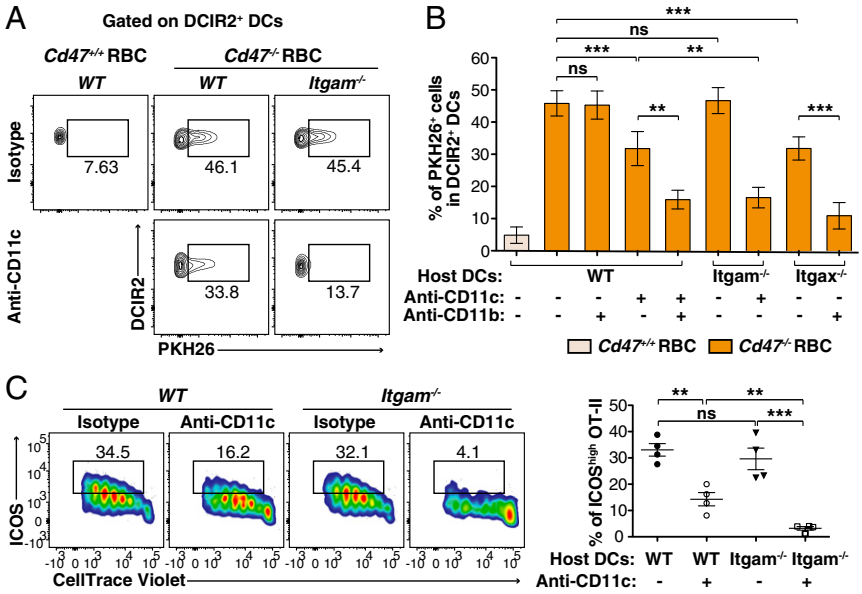

Fig. 4. CD11b is redundantly involved in CD47-deficient cell uptake in the absence of CD11c to support Tfh cell responses. (*A*) Representative FACS plots of PKH26 acquisition in gated DCIR2$^+$ DCs from mice of indicated genotypes pretreated with specified neutralizing antibodies for 1 h followed by i.v. immunization with PKH26-labeled *Cd47*$^{-/-}$ RBCs for 3 h. (*B*) Summary data of PKH26 acquisition in DCIR2$^+$ DCs from mice of indicated genotypes pretreated with specified neutralizing antibodies for 1 h followed by i.v. immunization with PKH26-labeled *Cd47*$^{+/+}$ or *Cd47*$^{-/-}$ RBCs for 3 h. Data are pooled from multiple experiments with each containing a subset of the groups with two to three mice per group. Mean ± SEM; $n = 3$ to 9. (*C*) Representative FACS plots and summary data showing the proliferation measured by violet tracer dilution and up-regulation of ICOS by transferred OT-II T cells in host mice of indicated genotypes pretreated with specified neutralizing antibodies followed by immunization with SRBC-OVA for 3 d. Data are pooled from two experiments with two to three mice per group. Statistical analyses were performed using the unpaired two-tailed Student's *t* test (**$P < 0.01$; ***$P < 0.001$; ns, not significant).

Cd47$^{-/-}$ cells resulted in defective T- and B-cell responses to CD47-deficient cell-associated antigen.

Even though several molecules have been shown to contain affinity for CD11c, none have been functionally verified in an in vivo setting, and the identity of the stimulatory ligand for CD11c on CD47-deficient cells is unclear. The fact that CD11b can partially compensate for CD47-deficient cell capture in the absence of CD11c suggests this potential ligand is likely shared by CD11b and CD11c, in accord with the sequence similarity of these integrin α-chains (20). Recently, the role for CD11b in phagocytosis of CD47-deficient cells has been established in BM-derived macrophages. In this case, it required the homotypic ligand/receptor SLAMF7 on both tumor cell targets and macrophages (27). However, *Cd47*$^{-/-}$ RBC uptake was unaffected in macrophages lacking SLAMF7, suggesting that SLAMF7 is not the dominant ligand on *Cd47*$^{-/-}$ RBCs (27). Another well-established common ligand for CD11b and CD11c is the iC3b fragment of complement C3 (17, 20, 28). However, DCIR2$^+$ DC capture of *Cd47*$^{-/-}$ RBCs and SRBCs, as well as DC activation, happens readily in C3-deficient recipients (*SI Appendix*, Fig. S6*A*). Although adhesion molecules ICAM1 and ICAM4 have also been shown to bind CD11c and CD11b in vitro (29–31), neither of them seems to be relevant. While mouse RBCs show no detectable expression of ICAM1 protein (*SI Appendix*, Fig. S6*B*), they do have enriched ICAM4 RNA expression compared with several other cell types tested (*SI Appendix*, Fig. S6*C*), consistent with other work (30, 31). Nevertheless, *Icam4*$^{-/-}$ *Cd47*$^{-/-}$ RBCs were captured by DCIR2$^+$ DCs indistinguishably from *Icam4*$^{+/+}$*Cd47*$^{-/-}$ RBCs (*SI Appendix*, Fig. S6*D*). We also considered the involvement of another putative CD11c ligand, heparin (19). However, mouse RBCs showed no discernible level of heparin sulfate on their surface (*SI Appendix*, Fig. S6*E*), and pretreating *Cd47*$^{-/-}$ RBCs with a mixture of heparinases I and III had no effect on their capture by splenic DCs (*SI Appendix*, Fig. S6*E*). Thus, future efforts are required to identify the ligand on CD47-deficient cells that triggers CD11c-dependent capture and DC activation.

Besides manifesting a defect in taking up CD47-deficent cells, DC Talin1-deficient mice were found to have decreased numbers of cDC2s. While lacking high-affinity CD11c/Itgb2 integrin was responsible for the defect of *Cd47*$^{-/-}$ cell capture in Talin1-deficient DCs, the decreased splenic cDC2 compartment in DC Talin1-deficient mice was not attributable to the lack of functional CD11c (*SI Appendix*, Fig. S5*B*). It is likely that other members of the integrin family were responsible for the observed cDC2 deficiency. The function of integrins in lymphocyte retention in the spleen, especially shear-exposed regions, has been established before (32). In agreement with the potential involvement of other integrins in the splenic retention of cDC2, the cDC2 reduction in DC Talin1-deficient mice was only observed in the spleen but not in peripheral lymph nodes where they have much less shear exposures.

The roles for DCs and adaptive immunity in mediating the therapeutic efficacy of CD47 blockade therapy are increasingly being appreciated. CD8 DCs and CD8 T cells have been shown to play important roles in such antitumor responses (13, 33). Our identification of the role for CD11c in DCIR2$^+$ DC CD47-deficient cell capture and activation to prime Tfh and B-cell responses may provide insights into the functional mechanism of anti-CD47 therapy. Activation of CD4 T-cell and antibody responses during CD47 antagonism might also contribute to the antitumor effect. It will be of interest, in future studies, to test whether CD11c is involved in tumor rejection in response to SIRPα–CD47 blockade.

Materials and Methods

Mice. C57BL/6-Ly5.1 (strain code 564) mice of 6 wk to 8 wk of age were purchased from the National Cancer Institute at Charles River. *CD47*$^{-/-}$, *Itgb1*$^{fl/fl}$, *Itgb2*$^{-/-}$, *Itgb7*$^{-/-}$, *Itgam*$^{-/-}$, Zbtb46-DTR (zDC-DTR), and *C3*$^{-/-}$ mice were purchased from Jackson Laboratory. *Tln1*$^{fl/fl}$, *Itgax*$^{-/-}$, *Itgb3*$^{-/-}$, and *Cd11c-cre* mice were provided by K. Ley, La Jolla Institute for Allergy and

Immunology; H. Wu, Baylor College of Medicine; and D. Sheppard and C. A. Lowell, University of California, San Francisco, respectively, and have been described before (22, 26, 34, 35). HEL-specific Hy10 and OVA-specific OT-II TCR transgenic mice were from an internal colony (3).

Icam4$^{-/-}$ mice were recovered from cryopreserved sperm from a previously described line (36) and were on a mixed B6/FVB strain background. For BM chimeras, mice were lethally irradiated by exposure to 900 rads to 1,100 rads of γ-irradiation in two doses 3 h apart, and donor BM cells (2×10^6 to 5×10^6) were i.v.-injected through tail veins. Chimeric mice were analyzed 6 wk to 10 wk after reconstitution. Procedure for DC ablation in zDC-DTR mix BM chimeras has been described before (23). Mice were housed in a specific pathogen-free environment in the Laboratory Animal Research Center at UCSF, and all animal protocols were approved by the UCSF Institutional Animal Care and Use Committee.

Adoptive Transfer and Immunizations. For analysis of CD4$^+$ T-cell responses, CellTrace Violet (ThermoFisher)-labeled splenocytes containing 5×10^5 OT-II T cells were adoptively transferred into mice through i.v. injection. For analysis of B-cell responses, splenocytes containing 3×10^5 HEL-binding Hy10 B cells were i.v.-transferred. Immunizations were conducted 1 d after adoptive transfer.

For CD47-deficient cell immunizations, RBCs (2×10^8), thymocytes (5×10^7), or BM cells (1.5×10^7) were collected from *Cd47*$^{-/-}$ and littermate control mice, labeled with PKH26 red fluorescent dye (Sigma-Aldrich), and i.v.-injected into recipient mice. For SRBC immunizations to induce T- and B-cell responses, mice were i.p.-injected with 3×10^8 to 4×10^8 SRBC (Colorado Serum), OVA-conjugated SRBCs, or HEL-conjugated SRBCs. Procedures for conjugating OVA or HEL to SRBC have been previously described (14, 25). For soluble OVA immunization, mice were i.v.-injected with 10 μg of OVA protein (Sigma-Aldrich) with 1 μg of LPS (O111:B4; Sigma-Aldrich). HKLM was prepared as described before (25, 37), and 1×10^9 HKLM were i.v.-injected into recipient mice.

Immunohistochemistry. For immunohistochemistry staining of DCIR2 and IgD, spleens were frozen in optimal cutting temperature compound. Cryosections of 7 μm were dried, fixed, stained, and imaged as previously described (23).

Immunohistochemistry images of DCIR2$^+$ DC positioning were quantified using ImageJ (v1.51s). After images were opened in ImageJ, freehand selection tool was used to outline a white pulp cord and the corresponding B–T border. The outlined regions were then saved in the ROI manager. ImageJ IHC toolbox was used to isolate DCIR2 staining signal and make a binary black and white image, with DCIR2 signal in black. For each outlined region, the total amount of black pixels representing DCIR2 signal was quantified. The ratio of the black pixels in the T zone to the entire white pulp cord was then calculated to approximate the proportion of DCs that has migrated from the bridging channel to the T zone.

In Vivo Antibody Blockade. For antibody blockade experiments, mice were i.v.-injected with neutralizing antibodies 1 h before immunization. In some of the OT-II T-cell experiments, mice were treated with antibodies again 2 d after immunization. The following antibodies were used to antagonize the function of different integrin subunits: 30 μg of anti-CD11c (N418; Biolegend), 100 μg of anti-CD11b (M1/70; Biolegend), or 100 μg of anti-CD11a (M17/4; Bio X cell).

Statistical Analysis. Statistical analyses were performed using Prism software (v5.0; GraphPad). Statistical significance was determined by the unpaired Student's *t* test (ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). In statistical graphs, points indicate individual mice, and results represent the mean ± SEM unless indicated otherwise.

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