Supplemental materials and methods

Sheep and mouse red blood cells conjugation

Sheep blood was obtained from Colorado Serum Company (Denver, CO) and mouse and human blood were always harvested freshly from healthy donors. For conjugation of RBCs with PKH26 (Sigma), the procedure was conducted following the manual instruction with 20μM PKH26 per 500μl blood cells in 1ml conjugation buffer. For RBC-OVA conjugation, chicken ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO) was first cross-linked with hen egg lysozyme (HEL) (Sigma-Aldrich) using glutaraldehyde (Sigma-Aldrich) and HEL-OVA was then conjugated to RBCs with HEL as a linker protein (OVA protein conjugated poorly to RBCs as compared to HEL; Yi T and Cyster JG., unpublished observation). RBCs were washed with PBS three times, mixed with HEL-OVA (50 μg/ml), crosslinked with EDCI (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, Sigma) for 30min, and washed three times to remove free protein. Conjugation efficiency was determined by anti-HEL (HyHEL9) FACS staining.

Flow Cytometry and antibody blockade

Antibodies were biotin, FITC, PE, APC, PerCP-Cy5.5, PE-Cy7, APC-Cy7, pacific blue, and BV605 conjugated anti-CD4 (GK1.5 or RM4-5), CD8 (53-6.7), TCRβ (H57-597), TCRVα2 (B20.1), CD80 (16-10A1), CD86 (GL-1), CCR7 (4B12), I-A^b (KH74), DCIR2 (33D1), B220 (RA3-6B2), GL7 (GL-7), Fas (DX2), IgD (11-26c.2a), ICOS (15F9), PD-1 (29F.1A12), and CXCR5 (L138D7) (all purchased from Biolegend, eBioscience or BD Pharmingen). For intracellular signaling molecule staining, splenocytes were mashed through 70μm cell strainers and instantly fixed with 1.5% *paraformaldehyde* (PFA) in RPMI with 5% fetal bovine serum for 10min. After centrifugation (1600rpm for 5min), the

cell pellet was permeabilized with 1ml cold methanol, added slowly while vortexing. Cells then incubated in cold methanol at -20°C overnight. Cells then were washed twice with PBS with 2% FBS, blocked with goat serum and anti-CD16/32, and stained with anti-pErk (197G2), anti-pCreb (87G3), anti-pS6 (D68F8), anti-pP38 (D3F9) (all obtained from Cell signaling; Beverly, MA). Data were acquired on a BD LSRII and analysis was performed using FlowJo (Treestar). Anti-Sirpα (P84) antibody, which previously reported to block Sirpα and CD47 interaction (Oldenborg et al., 2000), was purchased from Biolegend (San Diego, CA), and control rat IgG (anti-KLH) was purchased from BioXcell. For antibody blockade, mice were intravenously injected with 200μg through the tail veil.

For DC sorting in RNAseq analysis, Ebi2$^{+/-}$ mice were i.v. immunized with PBS or sheep red blood cells ($1x10^9$). 1 hour after immunization, spleens were meshed into single cell suspension through a 100-μm cell strainer in PBS buffers containing 2% FCS and 2 mM EDTA. CD4 DCs were first pre-enriched with anti-CD11c microbeads and MACS manual cell separation columns (Miltenyi Biotec) and further sorted based on surface markers of CD11c$^+$I-Ab$^+$CD4$^+$CD8$^-$. Cells were sorted twice on a FACSAira III with a 100-μm nozzle to purities of over 99%. Equal number of DCs ($5.0x10^5$) in SRBC or PBS immunized group was then snapped frozen immediately for RNA isolation.

Complement depletion

Mice were intraperitoneally injected with 4 units/mouse Cobra Venom Factor (Quidel; San Diego, CA) every 12 hrs for 36 hrs. Pilot experiment shows that the effect lasted up to 72 hrs.

Cell adoptive transfer and immunizations. For RBC immunization, RBCs were washed three times with sterile PBS and injected through the tail vein. SRBCs ($2x10^8$), human RBCs from 100µl blood, or mouse RBCs from 30 µl (or volumes indicated) of blood with or without prior PKH26 labeling were injected intravenously into recipient mice through the tail veil. To visualize cell proliferation, $0.5\text{-}1x10^6$ OT-I ($CD45.1^+CD45.2^+$) or OT-II ($CD45.1^+$) splenocytes labeled with CellTrace™ violet cell proliferation kit (Molecular Probes, Invitrogen) were injected intravenously into WT C57BL/6 ($CD45.2^+$) recipients; RBC-OVA conjugates or 33D1-OVA were intravenously injected 24hr after cell transfer. 33D1-OVA was produced by transfecting 293T cells with 33D1-OVA plasmid (kindly provided by Michel Nussenzweig, Rockefeller Univ., New York, NY) and purified through protein G affinity chromatography.

Reference:

Oldenborg, P.A., Zheleznyak, A., Fang, Y.F., Lagenaur, C.F., Gresham, H.D., and Lindberg, F.P. (2000). Role of CD47 as a marker of self on red blood cells. Science 288, 2051-2054.

Supplementary Table and Figures

Table S1. SRBCs upregulate splenic CD4$^+$ DC activation transcripts (refers to Figure 1). RNA-seq analysis of CD4$^+$ DCs from mice with or without SRBC immunization. Top 100 most upregulated genes upon 1hr SRBC immunization are listed. Average of two replicated reads is shown.

Figure S1. Activation of splenic CD4$^+$Sirpα^{hi} DCs by sheep and human RBCs (refers to Figure 1). (**A**) Mice were i.v. immunized with PBS or sonicated SRBC and analyzed 3hr later by flow cytometry for CD86, MHCII, and CCR7 in gated CD4$^+$ DCs. (**B**) Mice were i.v. immunized with PBS or indicated volumes of human RBCs and analyzed as in A. One representative experiment from four different human donors is shown. (**C**) WT or CD169DTR mice were treated with DT at days -4 and -1, mice were i.v. immunized with PBS or SRBCs on day 0 and then analyzed as in A. **(D)** Confocal microscope image of spleen section from a mouse that had been i.v. immunized with PKH26 (red) labeled SRBCs 30min earlier, stained for DCIR2 (33D1, green) and IgD (blue). **(E)** *Fcer1g*$^{-/-}$ mice were immunized and analyzed as in A. (**F**) Sirpα expression in gated CD4$^+$ and CD8$^+$ DCs. CD4$^+$ DCs that were in vivo pre-blocked with unconjugated Sirpα antibody were used as negative controls (background is similar to isotype control, data not shown). **(G)** *Sirpα* transcript in FACS-sorted CD4$^+$ DCs, CD8$^+$ DCs, or T cells. Hprt was used as internal control for normalization (mean ±SE, n=3).

Figure S2. Activation and capture of *Cd47*$^{-/-}$ RBCs by splenic CD4$^+$ DCs (refers to Figure 2). (A) Mice were i.v. immunized with WT or titrated amounts of *Cd47*$^{-/-}$ RBCs and analyzed 3hr later by flow cytometry for CD86 and CCR7 in gated CD4$^+$ DCs. **(B)** Mice were i.v. immunized with WT or 30 µl *Cd47*$^{-/-}$ RBCs isolated using the indicated

method and analyzed 3hr later for CD86 and CCR7 in gated $CD4^+$ DCs. **(C)** Mice were i.v. immunized with purified white-blood cells or red-blood cells from 40ul of blood and analyzed 3hr later as in B. **(D)** Mice were i.v. immunized with PKH26-labeled WT or *Cd47*$^{-/-}$ and analyzed 3hr later by flow cytometry for PKH26 in gated $CD4^+$ or $CD8^+$ DCs.

Figure S3. Activation of splenic $CD4^+$ DCs by Sirpα blockade and DC deficiency in *Cd47*$^{-/-}$ mice (refers to Figure 3). (A) Mice were i.v. injected with anti-Sirpα or control rat IgG2a and analyzed 6hr later by flow cytometry for CD86, MHCII, and CCR7 in gated $CD4^+$ and $CD8^+$ DCs. Mean fluorescence intensity (MFI) of gated $CD4^+$ or $CD8^+$ DCs relative to that of rat IgG control treated mice (mean±SE, n=3). **(B, C)** Flow cytometric analysis of splenocytes from WT or CD47$^{-/-}$ mice. **B,** representative flow cytometric pattern; **C,** mean (±SE) of splenic total DC, $CD4^+$ DC, and $CD8^+$ DC number in WT and *Cd47*$^{-/-}$ mice (n=3).

Figure S4. *Cd47*$^{-/-}$ RBCs, SRBCs and blockade of Sirpα augments $CD4^+$ T cells responses (refers to Figure 4). (A-C) $CD45.1^+$ OTII splenocytes were labeled with cell trace violet and adoptively transferred to WT mice. One day later, mice were i.v. immunized with or without 33D1-OVA followed 30min later by 30 µl of WT or *Cd47*$^{-/-}$ RBCs (A, B) or anti-Sirpα (C). 3 days after immunization, proliferation of OTII cells was visualized by violet tracer dye dilution. n=3 mice in each group. **(D)** $CD45.1^+$ OTII splenocytes were labeled with cell trace violet and adoptively transferred to WT mice and 1 day later, mice were immunized with WT or *Cd47*$^{-/-}$ RBC-OVA conjugate. 3 days post immunization, PD1 and CXCR5 on OTII cells was examined by flow cytometry. Numbers on gates indicate mean (±SE) frequencies for three mice. **(E)** $CD45.1^+$ OTII splenocytes were labeled with cell trace violet and adoptively transferred to WT or *Cd47*$^{-/-}$ mice and 1 day later mice were immunized with SRBC-OVA conjugate. 3 days post

immunization, PD1 and ICOS on OTII cells was examined by flow cytometry. Numbers on gates indicate mean (±SE) frequencies for three mice. **(F)** Flow cytometric analysis of Sirpα expression on $CD4^+$ or $CD8^+$ DCs from *Ebi2*$^{+/-}$ or *Ebi2*$^{-/-}$ mice.

Figure S5. Intrinsic requirement of Src family kinases in *Cd47*$^{-/-}$ RBC-mediated DC activation (refers to Figure 5). **(A)** *Fgr*$^{-/-}$, *Lyn*$^{-/-}$, and *Hck*$^{-/-}$ mice were i.v. immunized with WT or *Cd47*$^{-/-}$ RBCs and analyzed 3hr later by flow cytometry for CD86, MHCII, and CCR7 in gated $CD4^+$ DCs. **(B)** WT or *Hck*$^{-/-}$*Fgr*$^{-/-}$*Lyn*$^{-/-}$ mice were i.v. immunized with LPS and analyzed 3hr later as in A. **(C)** Flow cytometric analysis of splenic DCs from WT or *Hck*$^{-/-}$*Fgr*$^{-/-}$*Lyn*$^{-/-}$ mice. **(D)** Summary of WT:*Hck*$^{-/-}$*Fgr*$^{-/-}$*Lyn*$^{-/-}$ (HFL) mixed BM chimera data of the type shown in Figure 5D. Mean fluorescence intensity (MFI) of gated $CD4^+$ DCs is shown (mean±SE, n=4 mice). **(E)** Wild-type $CD45.1^+$ mice were reconstituted with mixed BM cells (1:1 ratio) from Zbtb46DTR mice ($CD45.1^+CD45.2^+$) and $CD45.2^+$ *Hck*$^{-/-}$*Fgr*$^{-/-}$*Lyn*$^{-/-}$ or WT mice. Chimeras were DT treated at days -4 and -1, immunized with PKH26-labeled WT or *Cd47*$^{-/-}$ RBCs on day 0 and analyzed 3 hr later by flow cytometry for PKH26, CD86, and CCR7 in gated CD4 DCs (n=3 mice). **(F)** Wild-type $CD45.1^+$ mice were reconstituted with fetal liver from *Syk*$^{-/-}$ mice. Chimeras were immunized with WT or *Cd47*$^{-/-}$ RBCs, or SRBCs and analyzed 3hr later as in A.

Figure S6. Integrin β 2 contributes to *Cd47*$^{-/-}$ RBC-mediated splenic CD4 DC activation (refers to Figure 6). **(A, B)** Mice reconstituted with mixed BM cells (1:1 ratio) from Zbtb46DTR and *Itgb2*$^{-/-}$ or WT mice were DT treated at days -6 and -3, immunized with PKH26-labeled WT or *Cd47*$^{-/-}$ RBCs on day 0 and analyzed 3 hr or 6 hr later (n=3 mice). **A,** Representative FACS analysis of PKH26, CD86, and CCR7 in gated CD4 DCs. **B,** Immunohistochemical staining of DCIR2 (33D1, brown) and IgD

(blue) on spleen sections taken at 6 hr. Black arrows highlight DCs in MZ bridging channels. **(C)** Wild-type CD45.1^{+} mice were reconstituted with *CD11c*$^{cre/+}$*Lrp1*$^{Flox/Flox}$ (DC-*Lrp1*$^{-/-}$) or control BM. Chimeras were immunized with 30µl WT or PKH26 labeled *Cd47*$^{-/-}$ RBCs, and analyzed 3hr later as in A. **(D)** OTII T cell response in mice reconstituted with *Itgb2*$^{+/+}$ and *Itgb2*$^{-/-}$ BM cells. GFP^{+} OTII CD4^{+} T cells were labeled with cell trace violet, adoptively transferred to the chimera mice and 1 day later mice were immunized with WT or *Cd47*$^{-/-}$ RBC-OVA conjugates. Frequency of OTII cells and violet tracer dilution were examined by flow cytometry. Numbers on gates indicate mean (±SE) frequencies for 3 mice. ** $p<0.01$, unpaired student t test.

Movie S1. Uptake of PKH26^{+} RBCs by splenic DCs at the MZ bridging channel (refers to Figure 2). Two photon microscopy of a vibratome sectioned spleen from a CD11c-yfp mouse that was i.v. immunized with PKH26-labeled *Cd47*$^{-/-}$ RBCs 20min earlier. YFP (green) and PKH26 (red). The image dimensions are 218, 215, 21 µm (x, y, z).

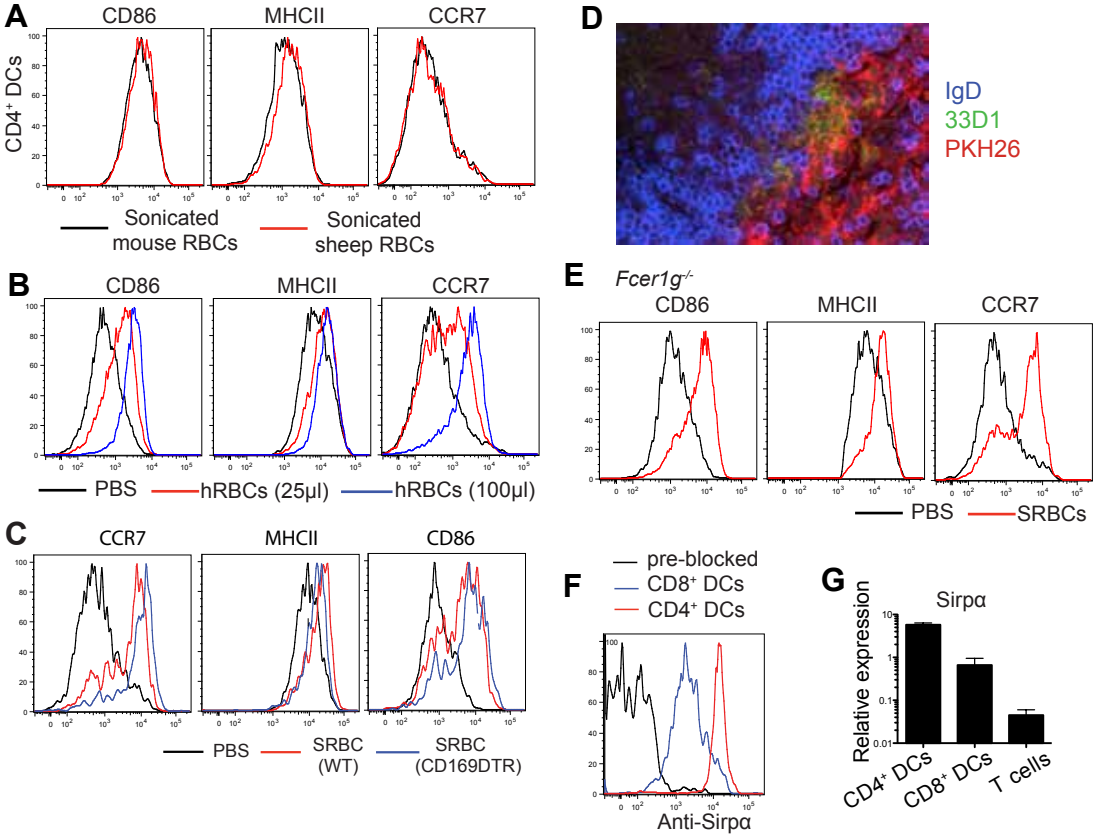
A
CD86
MHCII
CCR7
CD4⁺ DCs
Sonicated mouse RBCs
Sonicated sheep RBCs
B
CD86
MHCII
CCR7
PBS
hRBCs (25µl)
hRBCs (100µl)
C
CCR7
MHCII
CD86
PBS
SRBC (WT)
SRBC (CD169DTR)
D
IgD
33D1
PKH26
E
Fcer1g-/-
CD86
MHCII
CCR7
PBS
SRBCs
F
pre-blocked
CD8⁺ DCs
CD4⁺ DCs
Anti-Sirpα
G
Sirpα
Relative expression
CD4⁺ DCs
CD8⁺ DCs
T cells

Figure S1

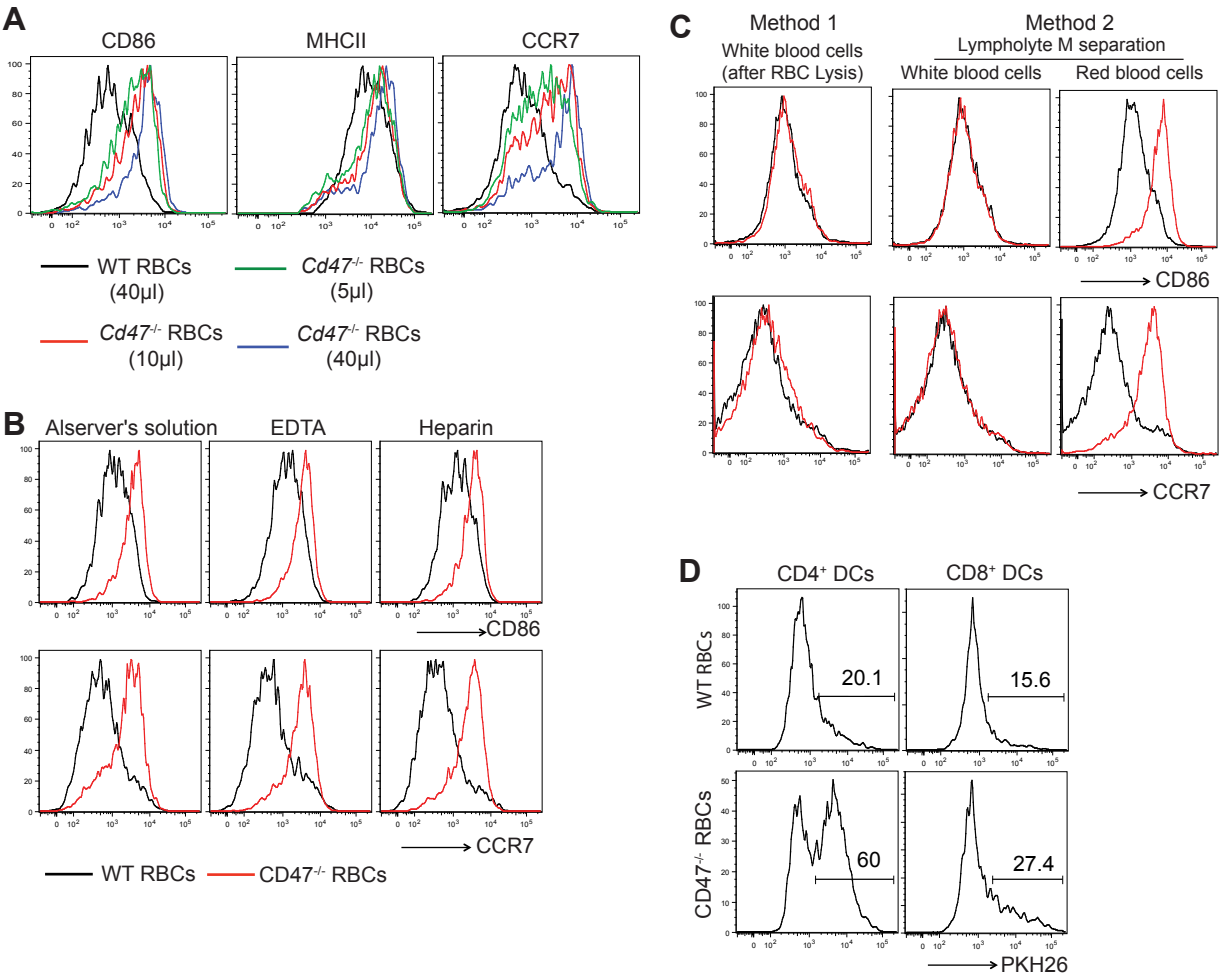
A
CD86
MHCII
CCR7
WT RBCs (40µl)
Cd47-/- RBCs (5µl)
Cd47-/- RBCs (10µl)
Cd47-/- RBCs (40µl)
B
Alserver's solution
EDTA
Heparin
CD86
CCR7
WT RBCs
CD47-/- RBCs
C
Method 1
White blood cells (after RBC Lysis)
Method 2
Lympholyte M separation
White blood cells
Red blood cells
CD86
CCR7
D
CD4+ DCs
CD8+ DCs
WT RBCs
CD47-/- RBCs
20.1
15.6
60
27.4
PKH26

Figure S2

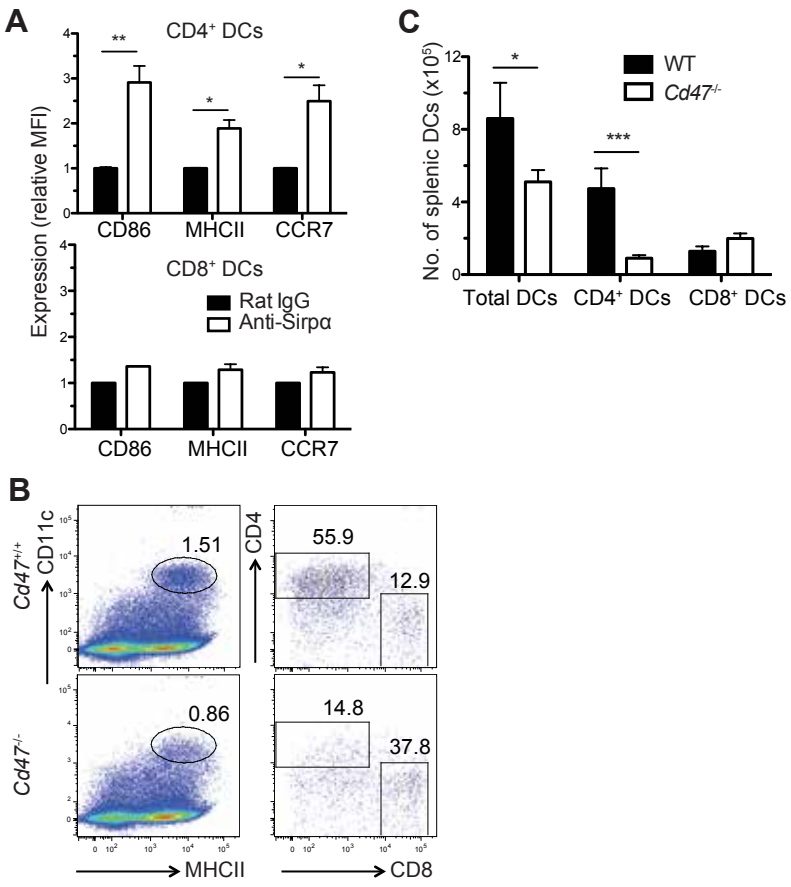
A
CD4⁺ DCs
Expression (relative MFI)
CD86
MHCII
CCR7
CD8⁺ DCs
Rat IgG
Anti-Sirpα
B
Cd47+/+
Cd47-/-
CD11c
CD4
1.51
55.9
12.9
0.86
14.8
37.8
MHCII
CD8
C
No. of splenic DCs (x10⁵)
WT
Cd47-/-
Total DCs
CD4⁺ DCs
CD8⁺ DCs

Figure S3

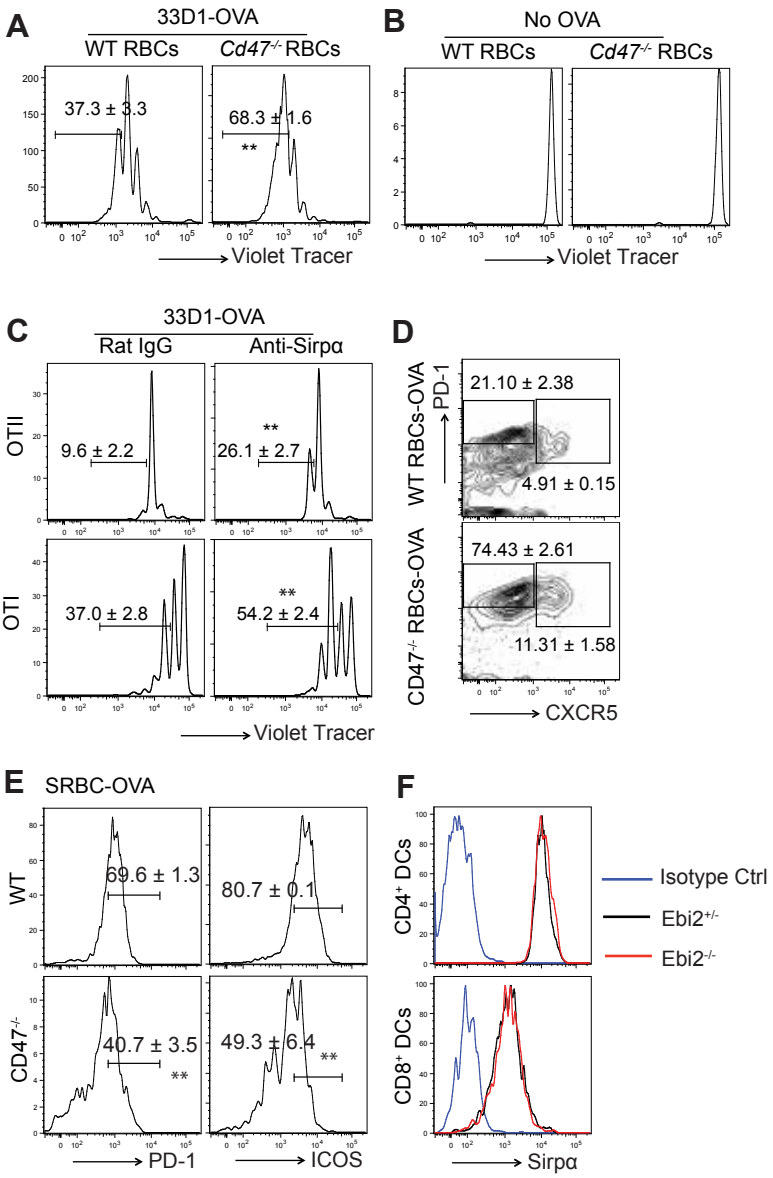
A
33D1-OVA
WT RBCs
Cd47-/- RBCs
37.3 ± 3.3
68.3 ± 1.6
**
Violet Tracer
B
No OVA
WT RBCs
Cd47-/- RBCs
Violet Tracer
C
33D1-OVA
Rat IgG
Anti-Sirpα
OTII
9.6 ± 2.2
**
26.1 ± 2.7
OTI
37.0 ± 2.8
**
54.2 ± 2.4
Violet Tracer
D
WT RBCs-OVA
PD-1
21.10 ± 2.38
4.91 ± 0.15
CD47-/- RBCs-OVA
74.43 ± 2.61
11.31 ± 1.58
CXCR5
E
SRBC-OVA
WT
69.6 ± 1.3
80.7 ± 0.1
CD47-/-
40.7 ± 3.5
**
49.3 ± 6.4
**
PD-1
ICOS
F
CD4+ DCs
CD8+ DCs
Isotype Ctrl
Ebi2+/-
Ebi2-/-
Sirpα

Figure S4

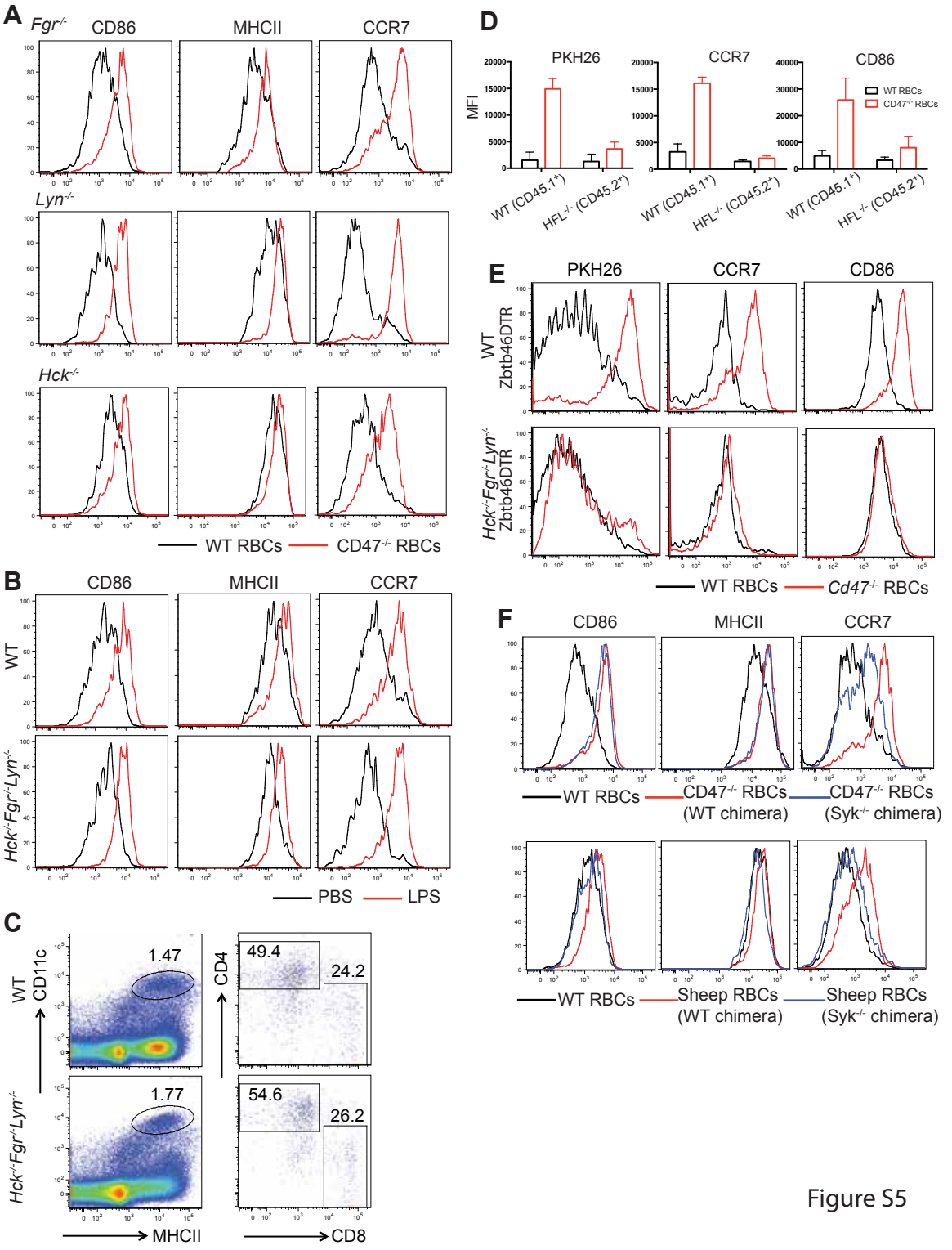
A
Fgr-/-
CD86
MHCII
CCR7
Lyn-/-
Hck-/-
WT RBCs
CD47-/- RBCs
B
WT
Hck-/-Fgr-/-Lyn-/-
PBS
LPS
C
WT
CD11c
1.47
49.4
24.2
CD4
Hck-/-Fgr-/-Lyn-/-
1.77
54.6
26.2
MHCII
CD8
D
PKH26
CCR7
CD86
MFI
WT RBCs
CD47-/- RBCs
WT (CD45.1+)
HFL-/- (CD45.2+)
E
PKH26
CCR7
CD86
WT Zbtb46DTR
Hck-/-Fgr-/-Lyn-/- Zbtb46DTR
WT RBCs
Cd47-/- RBCs
F
CD86
MHCII
CCR7
WT RBCs
CD47-/- RBCs (WT chimera)
CD47-/- RBCs (Syk-/- chimera)
WT RBCs
Sheep RBCs (WT chimera)
Sheep RBCs (Syk-/- chimera)

Figure S5

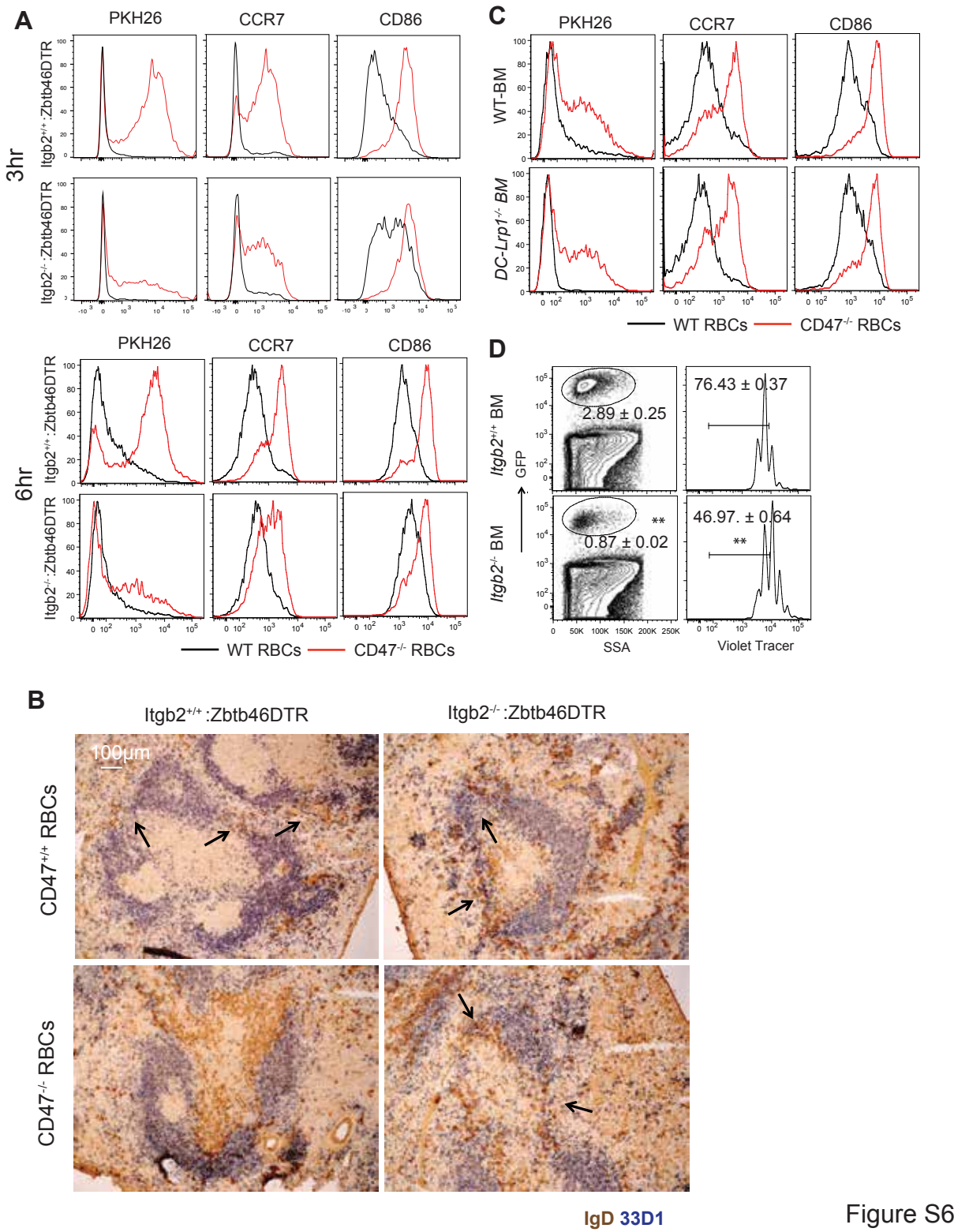
A
PKH26
CCR7
CD86
3hr
Itgb2+/+ :Zbtb46DTR
Itgb2-/- :Zbtb46DTR
6hr
WT RBCs
CD47-/- RBCs
C
WT-BM
DC-Lrp1-/- BM
D
Itgb2+/+ BM
Itgb2-/- BM
GFP
2.89 ± 0.25
76.43 ± 0.37
0.87 ± 0.02
46.97. ± 0.64
**
SSA
Violet Tracer
B
Itgb2+/+ :Zbtb46DTR
Itgb2-/- :Zbtb46DTR
100μm
CD47+/+ RBCs
CD47-/- RBCs
IgD 33D1

Figure S6