CRISPR/Cas9-mediated knockout of CD47 causes hemolytic anemia with splenomegaly in C57BL/6 mice

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CD47 (integrin-associated protein), a multi-spanning transmembrane protein expressed in all cells including red blood cells (RBCs) and leukocytes, interacts with signal regulatory protein α (SIRPα) on macrophages and thereby inhibits phagocytosis of RBCs. Recently, we generated a novel C57BL/6j CD47 knockout (CD47−/− hereafter) mouse line by employing a CRISPR/Cas9 system at Center for Mouse Models of Human Disease, and here report their hematological phenotypes. On monitoring their birth and development, CD47−/− mice were born viable with a natural male-to-female sex ratio and normally developed from birth through puberty to adulthood without noticeable changes in growth, food/water intake compared to their age and sex-matched wild-type littermates up to 26 weeks. Hematological analysis revealed a mild but significant reduction of RBC counts and hemoglobin in 16 week-old male CD47−/− mice which were aggravated at the age of 26 weeks with increased reticulocyte counts and mean corpuscular volume (MCV), suggesting hemolytic anemia. Interestingly, anemia in female CD47−/− mice became evident at 26 weeks, but splenomegaly was identified in both genders of CD47−/− mice from the age of 16 weeks, consistent with development of hemolytic anemia. Additionally, helper and cytotoxic T cell populations were considerably reduced in the spleen, but not in thymus, of CD47−/− mice, suggesting a crucial role of CD47 in proliferation of T cells. Collectively, these findings indicate that our CD47−/− mice have progressive hemolytic anemia and splenic depletion of mature T cell populations and therefore may be useful as an in vivo model to study the function of CD47.

Keywords: CRISPR/Cas9, CD47, hemolytic anemia, splenomegaly

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Immune system recognizes self and non-self using surface markers. CD47, also known as Integrin-Associated Protein (IAP), is a glycoprotein which ubiquitously exists on the surface of various cells including red blood cells (RBCs). CD47 functions as a “marker of self” on RBCs which prevents macrophages from phagocytosing self-RBCs [1-4]. Also, it is a potential immunoregulatory molecule in integrin signaling as demonstrated in downregulation of T cell activation and IL-12 production by inactivating CD47 using thrombospondin-1 (TSP-1) or anti-CD47 monoclonal antibodies [5-8]. In addition, loss of CD47 in mice impaired resistance to E. coli

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injection by causing delay of polymorphonuclear
leukocyte migration to the site of infection, indicating its
role in cell migration [3]. Due to its role in immune
response, CD47 has been frequently knocked out with
other immunity related genes such as Recombination
Activating Gene-2 (Rag-2) and γc to create immune
deficient animal models for transplantations studies [9,
10].

Generally, senescent RBCs are destroyed by splenic
macrophages when they reach approximately 40-day-old
[11]. However, destruction of RBCs in an early stage
may occur in several conditions such as autoimmune
disorders, genetic defects, drugs and red blood cell
membrane instability, resulting in anemia. There are
several types of anemia such as iron deficiency anemia,
thalassemia, aplastic anemia, hemolytic anemia, sickle
cell anemia and pernicious anemia. Among these, lack of
CD47 expression in non-obese diabetic (NOD) mice has
been associated with severe autoimmune hemolytic
anemia, suggesting a crucial role of CD47 in maintaining
the physiological levels of RBCs [12].

In this study, we report a novel CD47 knockout (KO)
mouse model (C57BL/6J-C\textsuperscript{D47}\textsuperscript{−/−}/Korl, CD47
−/− mice hereafter) that we generated using a CRISPR/Cas9
system at Center for Mouse Models of Human Disease
(CMHD), and their hematological phenotypes. We found
hemolytic anemia in 16-week-old CD47−/− mice, which
was aggravated in 26 weeks. In addition, our CD47−/− mice had a defective immune system due to reduction of
mature T cell populations in the spleen. These findings
confirm the role of CD47 in blood cells, indicating that
our CD47−/− mice may serve as a reliable platform to
study the function of CD47.

Materials and Methods

Generation of CD47\textsuperscript{−/−} mice and measurement of
major physiological parameters

CD47\textsuperscript{−/−} mice were generated in the C57BL/6J
background using a CRISPR/Cas9 system as previously
described [13]. For this study, heterozygous breeding
pairs were used to produce CD47\textsuperscript{−/−} mice and their
wildtype (WT) littermates (n=7-9 per group). Ear tissues
were used for genotyping in a conventional PCR with
specific primers; forward; 5’-GGT CGG TCG TTT CCC
TTG AA-3’ and reverse; 5’-GAT CCC CGA GCC ACT
CAC-3’. Mice were housed in an AAALAC International
accredited specific pathogen free (SPF) facility (#001169)
at Seoul National University Hospital under standard
housing conditions 12 h dark/light cycle, 40-60%
relative humidity and 22±2°C with free access to food
(LabDiet 5002 Certified Rodent Diet, PMI Nutrition
International, USA) and water. All experiments were
approved by the Institutional Animal Care and Use
Committee in Seoul National University Hospital and
animals were maintained in accordance with the Guide
for the Care and Use of Laboratory Animals [14].

From 4 weeks after birth to 26 weeks, body weight and
food/water intake of mice were measured once a week
with daily observation of clinical signs. Measurement of
food and water intake was carried out in the day cycle
and calculated as the amount consumed per mouse
during 24 hours.

Hematology, serum biochemistry and urinalysis

Hematology, serum biochemistry and urinalysis were
carry out as described [15]. Briefly, at the age of 16 and
26 weeks, all mice were deeply anesthetized with
isoflurane and peripheral blood was collected from the
abdominal vein in a K\textsubscript{2}EDTA contained tube (BD
Microtainer, USA) for hematologic analysis using an
ADVIA 2021i (Siemens Healthcare, USA). Serum
biochemistry and urinalysis were carried out using a
Hitachi 7070 (Hitachi, Tokyo, Japan) and a CLINTEK
Adventus analyzer (Siemens Healthcare), respectively.

Histopathology

At necropsy, gross examination was performed for all
organs. Then, major organs, except for eyes, testis and
epididymis which were fixed in Davidson solution and
Bouin’s solution, respectively, were fixed in 10% neutral
buffered formalin and embedded in paraffin wax after
dehydration and clearing process. After sectioning in a
thickness of 4-6 \(\mu\text{m}\), tissues were stained with hematoxylin
and eosin. To examine the levels of intracellular
accumulation of iron in the spleens, we stained the tissue
using Prussian blue reaction. Briefly, the paraffin-
embedded spleen was sectioned and re-hydrated after
deparaffinization. The tissue section was stained in
freshly prepared 5% potassium ferrocyanide (Sigma
Aldrich) in 10% HCl for 20 min and then counterstained
with neutral red (Sigma Aldrich) for 5 minutes. All
stained tissues were observed under a microscope
(BX61, Olympus).
Flow cytometry

Cells were isolated from the spleens, bone marrow and thymuses of 16-week-age WT and $CD47^{-/-}$ mice (male; $n=3$ and female; $n=3$ per genotype) and stained with antibodies for flow cytometric analysis as described [15]. In this study, we used V450-anti-CD3e (500A2), FITC-anti-CD4 (RM4-5), APC-anti-CD5 (53-7.3), BV605-anti-CD8a (53-6.7), Alexa700-anti-CD19 (1D3), and PerCP-anti-CD45R (RA3-6B2) to label distinct lymphocyte populations. All antibodies employed in this study were from BD Bioscience and used at 1:200 except anti-CD4 antibody (1:500). After staining and washing, 20,000 cells were analyzed using a FACS Calibur (BD).

Statistics

All data were expressed as mean±SD, and statistical analysis was performed using a two-tailed $t$ test in a SPSS software (version 22.0). $P<0.05$ was considered to be statistically significant.

Results and Discussion

Normal development of $CD47^{-/-}$ mice

To evaluate the role of CD47, we generated a novel $CD47^{-/-}$ mouse line by introducing a double-strand DNA break of $CD47$ exon 1 using a CRISPR/Cas9 system and inducing non-homologous end joining repair, which resulted in a deletion of 26 base pairs which created a frameshift mutation and a premature stop codon (Figure 1A).

To characterize the phenotype of $CD47^{-/-}$ mice, we first examined their development with monitoring of main physiological parameters including sex ratio, genotype, body weight gain, food and water intake for 26 weeks. At birth, there were no changes in the sex ratio of littermates (male; $n=45$, female; $n=43$ out of 88 siblings; $CD47^{-/-}$ male; $n=13$, $CD47^{-/-}$ female; $n=12$ out of 25 $CD47^{-/-}$ mice), and the ratio of genotypes of mice born from $CD47^{+/+}$ parents was 1:1.6:0.8 for WT, $CD47^{+/+}$ and $CD47^{-/-}$, indicating a Mendelian inheritance.
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For 26 weeks, body weight gain and food/water consumption of CD47−/− mice was comparable to WT (Figure 2), suggesting that loss of the CD47 gene did not affect the postnatal development.

Progressive anemia in CD47−/− mice

When peripheral blood was collected from 16-week-old mice and analyzed, male CD47−/− mice showed a significant reduction of RBC counts (11.4 %, *P<0.01) and hemoglobin (10.1%, *P<0.01) at the age of 16 weeks, while no abnormality was observed in the blood of female CD47−/− mice (Figure 3A). The anemia-like feature observed in male CD47−/− mice became more evident at 26weeks with increase of reticulocytes (136.2%, *P<0.01) and MCV (102.5%, *P<0.05) in...
addition to the reduction of RBC counts (13.1%, \(P<0.01\)), hemoglobin (10.9%, \(P<0.01\)) and hematocrit (10.7%, \(P<0.01\)) as shown in Figure 3B. 26-week-old female \(CD47^{-/-}\) mice also showed similar changes in the parameters for erythrocytes; reduction of RBC counts (8.0%, \(P<0.05\)), hemoglobin (11.1%, \(P<0.01\)) and hematocrit (7.5%, \(P<0.05\)) with increased reticulocytes (116.0%, \(P<0.05\)). These findings indicate the development of anemia in our \(CD47^{-/-}\) mice, which is progressively aggravated over aging.

Serum biochemistry (Table 1) and urinalysis (data not shown) showed no changes between \(CD47^{-/-}\) mice and their WT littermates, suggesting that lack of \(CD47\) may not affect the function of other organs, especially liver and kidney.

**Splenomegaly with intracellular iron accumulation in \(CD47^{-/-}\) mice**

Based on the function of \(CD47\) in RBCs, anemia observed in our \(CD47^{-/-}\) mice was possibly caused by

### Table 1. Serum biochemistry of \(CD47^{-/-}\) mice

| Parameters | WT male (n=7) | \(CD47^{-/-}\) male (n=7) | WT female (n=9) | \(CD47^{-/-}\) female (n=8) |
|------------|---------------|---------------------|-----------------|---------------------|
| BUN (mg/dL) | 23.3±9.5     | 22.9±3.4            | 18.6±2.9        | 22.8±4.9           |
| Chol (mg/dL) | 112.7±23.4   | 119.9±8.4          | 86.1±16.8       | 81.9±15.0          |
| T.protein (g/dL) | 4.5±0.4    | 4.4±0.4          | 4.5±0.3        | 4.6±0.3           |
| Albumin (g/dL) | 1.6±0.1    | 1.7±0.1         | 1.7±0.1        | 1.7±0.1           |
| T.bil (mg/dL) | 0.05±0.02   | 0.06±0.04        | 0.08±0.04      | 0.04±0.04         |
| ALP (IU/L)   | 194.2±48.1  | 211.4±32.6       | 288.3±70.4     | 310.3±53.3        |
| AST (IU/L)   | 83.7±64.1   | 111.7±104.5      | 110.1±95.0     | 113.5±34.6        |
| ALT (IU/L)   | 32.8±15.7   | 42.1±31.6        | 51.1±58.2      | 44.6±16.1         |
| \(\gamma\)GT (IU/L) | -0.5±1.2 | -0.6±0.8        | 0.2±0.7        | -0.3±0.9          |
| Creatinine (mg/dL) | 0.28±0.03 | 0.29±0.06       | 0.25±0.12      | 0.28±0.10         |
| TG (mg/dL)   | 52.0±25.3   | 51.7±24.5        | 23.6±6.4       | 37.6±25.9         |
| Glu (mg/dL)  | 251.0±43.5  | 262.4±17.9       | 275.8±38.3     | 264.6±38.9        |

**Abbreviation.** Blood urea nitrogen (BUN), cholesterol (Chol), total protein (T.protein), total bilirubin (T.bil), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma (\(\gamma\))-glutamyl transferase (\(\gamma\)GT), triglyceride (TG), glucose (Glu).

Figure 4. Increased size and weight of spleens in \(CD47^{-/-}\) mice. Representative images and bar graphs show increased size and weight of spleens in the 16 week-old (A) and 26 week-old (B) \(CD47^{-/-}\) mice compared to their respective wild-type littermates. The graphs are expressed as a percentage of organ weight/body weight (black bars, WT male; dark gray bars, \(CD47^{-/-}\) male; light gray bars, WT female; and white bars, \(CD47^{-/-}\) female mice). Scale bar; 5 mm. *\(P<0.05\) and **\(P<0.001\).
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increased hemolysis. As spleen is commonly enlarged in hemolytic anemia due to accumulation of RBCs and macrophages [16,17], we examined the organs of CD47−/− mice with a particular focus on their spleens. While no changes were found in other organs of CD47−/− mice, spleens of CD47−/− mice were found to be significantly larger than those of WT littermates (Figure 4, Table 2). When the organ per body weight ratio was calculated, both genders of 16-week-old CD47−/− mice exhibited enlarged spleens (Figure 4A; 169.3% increase compared to WT male, 139.0 % for female mice, P<0.05 for both).

In line with the progression of anemia observed in hematological analysis, splenomegaly was consistently observed in both genders of 26 week-old CD47−/− mice (Figure 4B; 143.5 % increase in male, P<0.05 and 161.8 % in female, P<0.001). Except for the spleen, no other organs in CD47−/− mice showed notable changes (Table 2).

When the spleen was histopathologically examined, cells containing brown-gold pigments were more frequently observed in CD47−/− mice compared to WT mice (Figures 5A, 5B). As loss of CD47 was previously associated with increased destruction of RBCs by macrophages, these pigments were likely hemosiderins derived from increased degradation of iron-containing hemoglobins in RBCs and therefore we visualized intracellular iron in the tissue using Prussian blue reaction to determine the identity of the pigments. Staining of iron revealed noticeable increase in the number of dark blue-stained cells with a tendency of higher intracellular staining levels in CD47−/− mice than WT mice (Figures 5C, 5D), indicating the increased intracellular accumulation of hemosiderns. It is tempting to postulate that these hemosiderin-containing cells were macrophages due to their function in hemolysis, but this proposition may need to be confirmed. There are several types of anemia reported in humans. Among them, the anemia identified in our CD47−/− mice is likely hemolytic based on the following observations; CD47−/− mice showed concurrent reduction of RBC counts, hemoglobin and hematocrit with increased reticulocytes, indicating loss of mature RBCs with increased release of immature RBCs into the blood as a compensatory response. Although the anemic feature was not evident in 16-

Table 2. Absolute and relative weight of major organs of 26-week-old CD47−/− mice

| Organ                  | WT male (n=7) | CD47−/− male (n=7) | WT female (n=9) | CD47−/− female (n=8) |
|------------------------|---------------|--------------------|-----------------|----------------------|
| Body weight (g)        | 38.03±4.56    | 35.03±3.94         | 24.67±3.11      | 23.29±2.75           |
| Liver (g)              | 1.71±0.25     | 1.69±0.28          | 1.04±0.18       | 1.04±0.14            |
| (g%)                  | 4.53±0.62     | 4.79±0.31          | 4.19±0.24       | 4.46±0.29            |
| Spleen (g)             | 0.09±0.01     | 0.11±0.02          | 0.08±0.01       | 0.13±0.02***         |
| (g%)                  | 0.23±0.05     | 0.33±0.08          | 0.34±0.02       | 0.55±0.08***         |
| Kidney (right) (g)     | 0.20±0.03     | 0.18±0.03          | 0.13±0.01       | 0.12±0.01            |
| (g%)                  | 0.53±0.08     | 0.52±0.06          | 0.54±0.05       | 0.52±0.05            |
| Kidney (left) (g)      | 0.20±0.03     | 0.18±0.02          | 0.13±0.02       | 0.12±0.01            |
| (g%)                  | 0.54±0.12     | 0.52±0.05          | 0.52±0.04       | 0.51±0.05            |
| Adrenal gland (g)      | 0.0013±0.0006 | 0.0014±0.0040      | 0.0032±0.0009   | 0.0027±0.0006        |
| (right) (g%)           | 0.0033±0.0014 | 0.0040±0.0014      | 0.0130±0.0027   | 0.0117±0.0022        |
| Adrenal gland (left) (g%) | 0.0012±0.0004 | 0.0015±0.0005      | 0.0033±0.0008   | 0.0035±0.0010        |
| Testis (right) (g)     | 0.12±0.02     | 0.11±0.01          | 0.0045±0.0008   | 0.0035±0.0010        |
| (g%)                  | 0.32±0.05     | 0.32±0.03          | 0.0187±0.0047   | 0.0132±0.0044        |
| Testis (left) (g)      | 0.11±0.01     | 0.11±0.01          | 0.0037±0.0012   | 0.0028±0.0009        |
| (g%)                  | 0.30±0.05     | 0.32±0.02          | 0.150±0.0048    | 0.12±0.0041          |
| Thymus (right) (g)     | 0.05±0.01     | 0.04±0.01          | 0.05±0.01       | 0.04±0.01            |
| (g%)                  | 0.13±0.01     | 0.11±0.01          | 0.19±0.02       | 0.19±0.03            |
| Heart (g)              | 0.15±0.02     | 0.15±0.02          | 0.12±0.01       | 0.12±0.02            |
| (g%)                  | 0.40±0.05     | 0.44±0.06          | 0.50±0.04       | 0.50±0.07            |
| Lung (g)               | 0.15±0.01     | 0.15±0.01          | 0.14±0.01       | 0.14±0.01            |
| (g%)                  | 0.40±0.06     | 0.43±0.06          | 0.58±0.06       | 0.60±0.10            |
| Brain (g)              | 0.46±0.03     | 0.46±0.02          | 0.47±0.01       | 0.48±0.01            |
| (g%)                  | 1.23±0.16     | 1.33±0.13          | 1.93±0.23       | 2.08±0.25            |

*P<0.05 and ***P<0.001 in comparison to respective gender-matching WT mice
week-old female mice, both genders of CD47−/− mice had enlargement of the spleen which continued to the age of 26 weeks with concurrent aggravation of hematological parameters regarding RBCs. Furthermore, the elevated destruction of RBCs was demonstrated by the increased number of hemosiderin-loaded cells with higher amount of hemosiderins accumulated in each cells of the CD47−/− spleens, strongly supporting our hypothesis. Despite these findings, our data however do not exclude the possibility of other types of anemia with similar features. Therefore, further investigation is warranted to characterize the type and clinical manifestation of the anemia in our CD47−/− mice.

The findings on the development of hemolytic anemia in our CD47−/− mice have not been reported in the previously generated CD47−/− mice [4], but were closely in line with AIHA in CD47−/− NOD mice [12] with several different features noted; CD47−/− NOD mice showed much greater reduction of hematocrit than our CD47−/− mice, while jaundice was only observed in CD47−/− NOD mice, suggesting a mild degree of hemolytic anemia in our mice. The mouse strains used in generating models (NOD vs. C57Bl/6) may have played a role in causing these discrepancies in manifestation of hemolytic anemia between the two mouse lines.

Reduction of T cells in the spleen of CD47−/− mice

Next, we examined changes in subpopulations of
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Lymphocytes including T and B cells in CD47−/− mice as inactivation of CD47 previously resulted in reduction of T cell populations in the spleen [18]. To assess the impact of CD47 deficiency on those lymphocytes, we performed flow cytometry on the cells isolated from 16-week-old mice spleens, thymuses and bone marrow. Flow cytometry analysis on splenic lymphocytes revealed a remarkable reduction of CD3−CD5−, CD3−CD4− and CD3−CD8− T cell populations (Figures 6A, 6C, P<0.01 for all), while no change was found in CD19−B220− B cell population. The number of CD19−B220− B cells from the bone marrow of CD47−/− mice was also comparable to WT (Figures 6B, 6D). Our findings recapitulated the crucial role of CD47-SIRPα interaction on T cell proliferation as similar findings on the reduction of CD4+ T cells have been reported in the spleen of the CD47 binding partner SIRPα-deficient mice [18]. Contrary to the reduction of T cells in the spleen, no change was detected in thymic T cell populations when compared to WT (Figures 6C, 6F), suggesting differential roles of CD47 in T cells depending on their developmental stage; CD47 functions in the proliferation of T cells which occurs in the spleen, but not at the stage of initial development through negative selection in the thymus [19].

In this study, we generated a novel CD47−/− C57BL/6J
mouse line using a CRISPR/Cas9 system and found a progressive hemolytic anemia as well as profound reduction of mature T cell populations in the spleen. Intriguingly, despite its ubiquitous expression, loss of CD47 mainly resulted in hematological phenotypes without causing embryonic lethality, developmental defects or any noticeable changes in the structure and function of other tissues, indicating its preferred function in blood cells. These results, however, do not exclude the possible role of CD47 in other types of cells and further investigation may be required to reveal cell-specific impact of CD47 deficiency. In conclusion, CRISPR/Cas9-mediated generation of CD47−/− mice confirmed the function of CD47 in the maintenance of RBC and T cell populations, demonstrating their usefulness as an in vivo platform to study the function of CD47.

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Conflict of interests The authors declare that there is no financial conflict of interests to publish these results.

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