stranger 1 (familiar side) versus stranger 2 (stranger side) in the mutants (Figure 3E; $t_{19} = 1.609$, $p = 0.1241$). In contrast, wild-type mice spent significantly more time around the familiar side than the stranger side (Figure 3E; $t_{19} = 2.643$, $p = 0.0160$). Genotype comparison revealed that the mutants spent significantly less time around the cage with stranger 2 (stranger side) than did the wild-type mice (genotype effect, $F_{1,38} = 5.192$, $p = 0.0284$). No significant genotype effect was detected on ratio of time spent with stranger 2 / total time spent with both cages (Mann-Whitney U-test, $p = 0.1762$). In CD47 KO mice, no significant differences were detected in time spent among the three chambers (Figure 3F, H; familiar side versus stranger side, $t_{19} = 0.068$, $p = 0.9467$; center vs. familiar side, $t_{19} = 0.046$, $p = 0.9639$; center vs. stranger side, $t_{19} = 0.051$, $p = 0.9601$). In contrast, wild-type mice spent significantly less time in the center chamber than in the other chambers (Figure 3F, H; familiar side versus stranger side, $t_{19} = 1.447$, $p = 0.1643$; center versus familiar side, $t_{19} = 3.546$, $p = 0.0022$; center versus stranger side, $t_{19} = -2.569$, $p = 0.0188$). Ratio of time spent in stranger 2 side to center area for CD47 KO mice was smaller than that for wild type mice (Mann-Whitney U-test, $p = 0.0041$). The mutants also showed significantly greater values for total distance traveled than wild-type mice (Figure 3G; $F_{1,38} = 6.329$, $p = 0.0162$).

In an alternative version of the social interaction test in which two mice move freely and can have direct interactions in a novel environment for 10 min, no significant differences were detected between the genotypes in total duration of contacts (Figure 4A; $F_{1,18} = 1.060$, $p = 0.3169$), number of contacts (Figure 4B; $F_{1,18} = 0.268$, $p = 0.6113$), total duration of active contacts (Figure 4C; $F_{1,18} = 0.891$, $p = 0.3578$), mean duration per contact (Figure 4D; $F_{1,18} = 0.263$, $p = 0.6146$), or distance traveled (Figure 4E; $F_{1,18} = 0.641$, $p = 0.4337$). Social interaction was also monitored in the home cage over a 9-day period. There were no significant differences between the genotypes in the mean numbers of particles (Figure 4F; $F_{1,14} = 0.424$, $p = 0.5256$) or activity level (Figure 4G; $F_{1,14} = 0.101$, $p = 0.7558$). Thus, although no abnormalities in social behavior of the mutants were detected in the social interaction tests in which mice had direct contact under freely moving settings, significantly reduced social behavior was observed in Crawley's three-chamber social approach test. These findings suggest that a CD47 deficiency may induce mild impairments in sociability and social novelty preference in mice.

4. Normal Anxiety-like and Depression-like Behaviors in CD47 KO Mice

Anxiety-like behaviors of CD47 KO mice were evaluated in the open field, light/dark transition, and elevated plus-maze tests. In the open field test, CD47 KO mice tended to spend longer in the center area compared to wild-type mice over the entire experimental period (Figure 5A; 0–120 min, $F_{1,37} = 2.952$, $p = 0.0941$), and the mutants showed an increase in center time during the earlier part of the experimental period (Fig. 5A; 0–60 min, $F_{1,37} = 7.390$, $p = 0.0099$). Since increased center time was only seen in the earlier part of the experimental period, it is possible that a deficiency in CD47 may lead to decreased anxiety in a novel environment in these mice. On the other hand, there were no significant differences in total distance traveled (Figure 5B; $F_{1,37} = 0.285$, $p = 0.5969$), vertical activity (Figure 5C; $F_{1,37} = 0.101$, $p = 0.7522$), or stereotypic behavior (Figure 5D; $F_{1,37} = 1.588$, $p = 0.2155$). In the light/dark transition test, no significant differences were observed between the genotypes with respect to time spent in the light box (Figure 5E; $F_{1,38} = 1.594$, $p = 0.2144$) or number of transitions (Figure 5F; $F_{1,38} = 0.164$, $p = 0.6877$). In the elevated plus-maze test, there were no significant differences between genotypes in entries to the open arms (Figure 5G; $F_{1,38} = 0.038$, $p = 0.8462$), or time spent on the open arms (Figure 5H; $F_{1,38} = 0.494$, $p = 0.4864$). Thus, anxiety-like behaviors did not significantly differ between genotypes.

CD47 KO mice were also subjected to the tail suspension test to assess depression-like behavior. No significant effect of genotype was found for percent time immobile (Figure 6; $F_{1,37} = 0.341$, $p = 0.5628$). Thus, in the tail suspension test, in which phosphorylation of SIRPα is known not to be induced [21], no behavioral abnormalities were detected in CD47 KO mice.

5. Normal Fear and Spatial Memory in CD47 KO Mice

Finally, learning and memory of CD47 KO mice was assessed in the fear conditioning and Barnes maze tests. In the fear conditioning test, no significant differences between the genotypes were detected in the amount of freezing after footshocks in the conditioning phase (Figure 7A; $F_{1,37} = 1.361$, $p = 0.2507$). Contextual freezing of the mutants also did not significantly differ from that of wild-type mice 1 day after training (Figure 7A; $F_{1,37} = 0.044$, $p = 0.5853$). Freezing before or after the auditory cue as measured in an altered context was also not significantly different across the genotypes (Figure 7A; $F_{1,37} = 0.204$, $p = 0.6543$ [before tone]; $F_{1,37} = 0.039$, $p = 0.8451$ [after tone]). In the Barnes maze test, CD47 KO mice showed no significant differences in time required to reach the escape box (the 1st hole) (Figure 7B; $F_{1,37} = 1.443$, $p = 0.2374$) or number of errors (Figure 7C; $F_{1,37} = 0.276$, $p = 0.6027$) during the training session. There were also no significant differences between the genotypes in time spent around the hole where the escape box had been placed in probe tests performed either 24 h (Figure 7D; $F_{1,37} = 1.317$, $p = 0.2586$) or 2 weeks (Figure 7E; $F_{1,37} = 0.188$, $p = 0.6674$) after the last training session. These findings indicate normal memory acquisition and retention in the mutants. The present study thus failed to detect impairments of CD47 KO mice in learning and memory.

Discussion

In this study, we assessed the behavioral phenotypes of CD47 KO mice with our comprehensive behavioral test battery. We have summarized the behavioral phenotypes of CD47 KO mice, CD47 antisense-injected rats [22], and SIRPα mutant mice [21], as identified in the present and previous studies [23], in Table S1. While the present study revealed that the lack of CD47 did not lead to significant abnormalities in overall health, a few physical and behavioral characteristics were newly identified. First, in the startle response/PPI tests, while the acoustic startle response of CD47 KO mice did not significantly differ from that of their wild-type littermate mice, the mutants exhibited mild but significant reductions in PPI, suggesting that CD47 is involved in the regulation of sensorimotor gating in mice.

Second, in Crawley's three-chamber social approach test, the mutants showed slight reductions in sociability and social novelty preference, indicating that CD47 is involved in the regulation of social behavior. On the other hand, in social interaction tests in which pairs of subject animals could interact directly in a novel environment or their home cage, no abnormalities were detected in either social behavior or locomotor activity of the mutants. It is possible that these discrepant findings in different social interaction assays are due to the assays' differential requirements for intact olfactory function. It is reported that when animals have olfactory impairment, abnormal social behaviors can be observed in Crawley's three-chamber social approach test, in which mice must spend time near animals that are isolated in wire cages and not directly accessible [27]. Therefore, the possibility cannot be

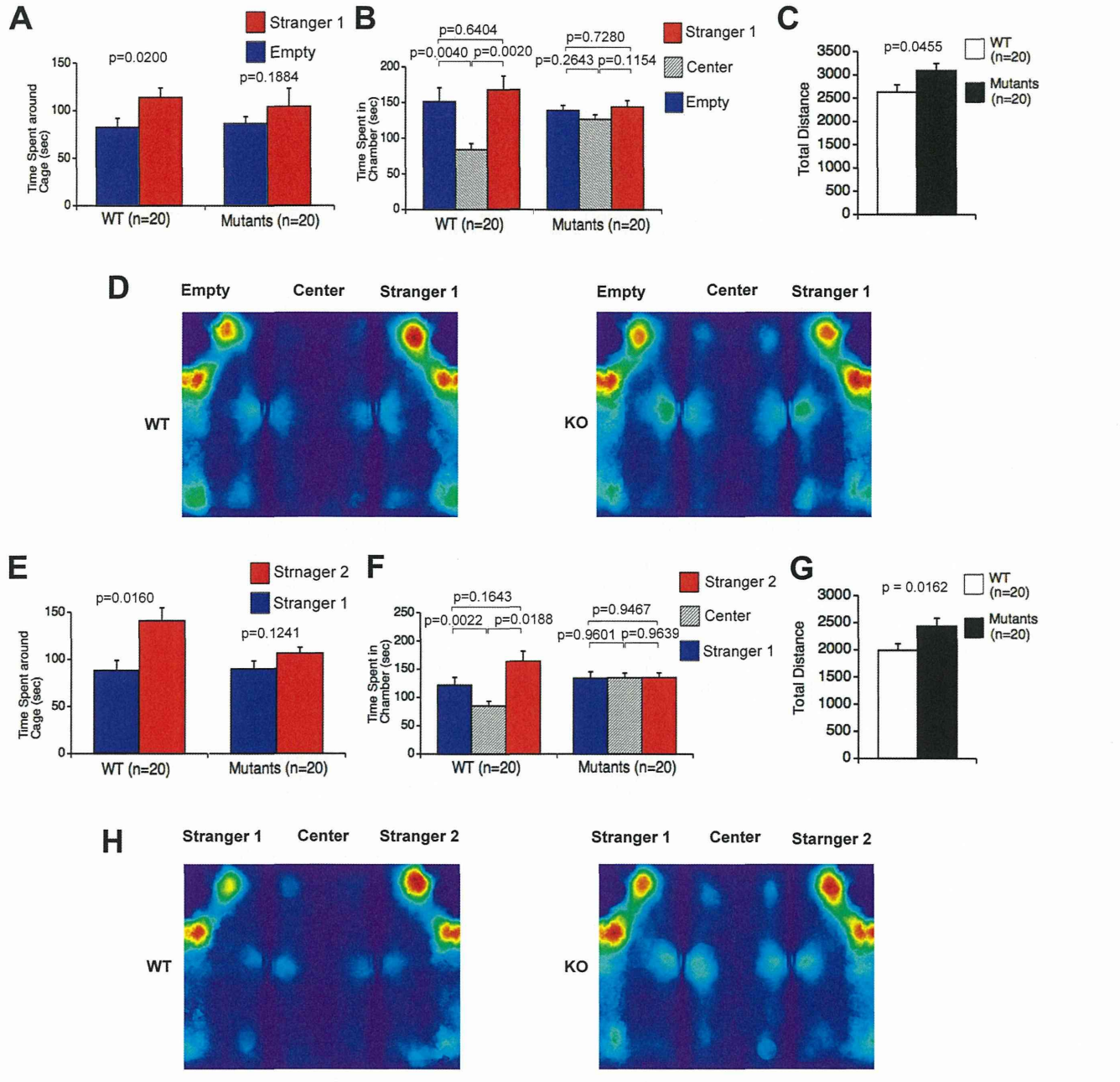

Figure 3. Abnormal social behavior of CD47 KO mice in Crawley's sociability and social novelty preference test. The sociability test was performed first (A–D). (A) CD47 KO mice did not show significant differences between time spent around cage with stranger 1 and time spent around the empty cage. By comparison, wild-type mice spent more time around the cage with the stranger 1 than around the empty cage. (B) CD47 KO mice displayed no significant differences in time spent among the three chambers, whereas wild-type mice spent less time in the center chamber than in the other two chambers. (C) Distance traveled by CD47 KO mice was significantly longer than that of wild-type mice. (D) All images of each mouse were superimposed, and averaged images for the traces of wild-type mice (left, WT) and CD47 KO mice (right, KO) in the sociability test are presented. Following completion of the sociability test, the social novelty preference test was carried out (E–H). (E) The mutants exhibited no significant differences between time spent around the cage with stranger 1 (familiar side) and time spent around the cage with stranger 2 (stranger side). However, wild-type mice spent significantly less time in the familiar side (stranger 1) than in the stranger side (stranger 2). (F) No significant differences were detected in time spent in the three chambers in CD47 KO mice, while wild-type mice spent less time in the center chamber than in the other chambers. (G) Distance traveled by CD47 KO mice was significantly longer than distance traveled by wild-type mice. (H) All images of each mouse were superimposed and averaged images for the traces of wild-type mice (left) and CD47 KO mice (right) in the social novelty preference test are shown. CD47 KO mice, n = 20; wild-type mice, n = 20.
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excluded that the decreased sociability and social novelty preference in this test were caused by an olfactory impairment in the mutant mice. Further behavioral or physiological analyses would be needed to address whether CD47 KO mice have normal olfaction.

Third, in the gait analysis the mutants demonstrated narrower stance widths of the front and hind paws, and narrower paw

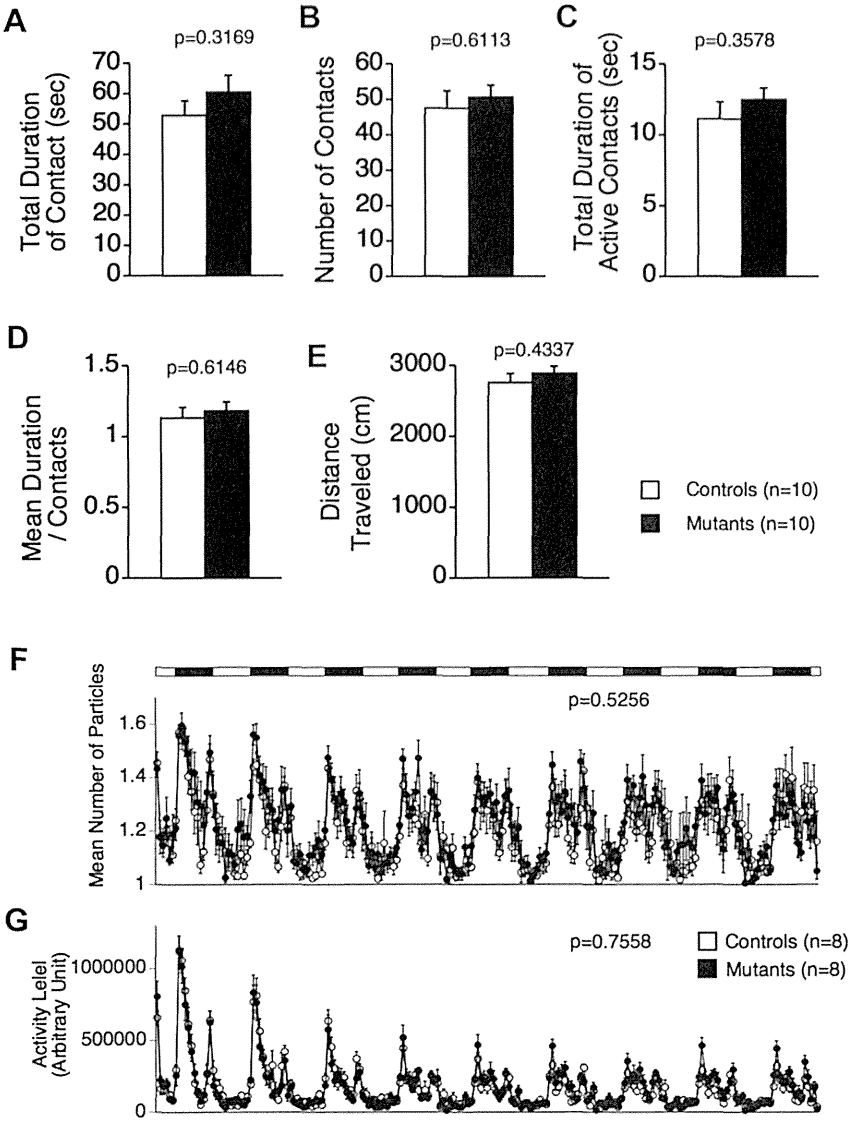

Figure 4. Normal social behaviors of CD47 KO mice in a novel environment and the home cage. (A–E) In the social interaction test performed in a novel environment, there were no significant differences between the genotypes in total duration of contacts (A), number of contacts (B), total duration of active contacts (C), mean duration per contact (D), or distance traveled (E). CD47 KO mice, n = 10 pairs; wild-type mice, n = 10 pairs. (F, G) Social interaction in the home cage was monitored over a 9-day period. There were no significant differences between the genotypes in the mean numbers of particles, i.e., whether 1 (mice together) or 2 (mice apart) subjects were detected (F) or activity level (G). CD47 KO mice, n = 8 pairs; wild-type mice, n = 8 pairs.
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placement angles for the hind paws. These findings indicate that CD47 is involved in motor coordination and that CD KO may lead to improved postural adjustments and stability [24,25]. Additional histological and electrophysiological analyses in brain areas such as the cerebellum, basal ganglia, and motor cortex, would be important to assess the role of CD47 in motor coordination in mice. Additionally, in the open field test, CD47 KO mice stayed significantly longer in the center of the field during the earlier part of the experimental period, but not over the entire experimental period, indicating that the mutants show decreased anxiety-like behavior in a novel environment. These results point to the possibility that CD47 is involved in the regulation of anxiety in a novel environment.

In the tail suspension test, CD47 KO mice exhibited no impairments in depression-like behavior. In our previous study, we revealed that forced swimming stress, but not tail suspension stress, led to CD47-dependent tyrosine phosphorylation of SIRPα in the brain of mice [21]. In an earlier study, both CD47 KO mice and SIRPα mutant mice displayed increased depression-like behaviors in the forced swimming test, while there were no changes in depression-like behavior of SIRPα mutant mice in the tail suspension test (Table S1) [21]. Our previous findings indicate that CD47 and SIRPα are not involved in the regulation of depression-like behaviors when stress does not cause tyrosine phosphorylation of SIRPα in the brain [21], and this is consistent with the present observation that CD47 KO mice demonstrated normal performance in a test that does not lead to CD47-dependent tyrosine phosphorylation of SIRPα [21]. It would be of interest to address whether tyrosine phosphorylation of SIRPα is also involved in the regulation of other behaviors.

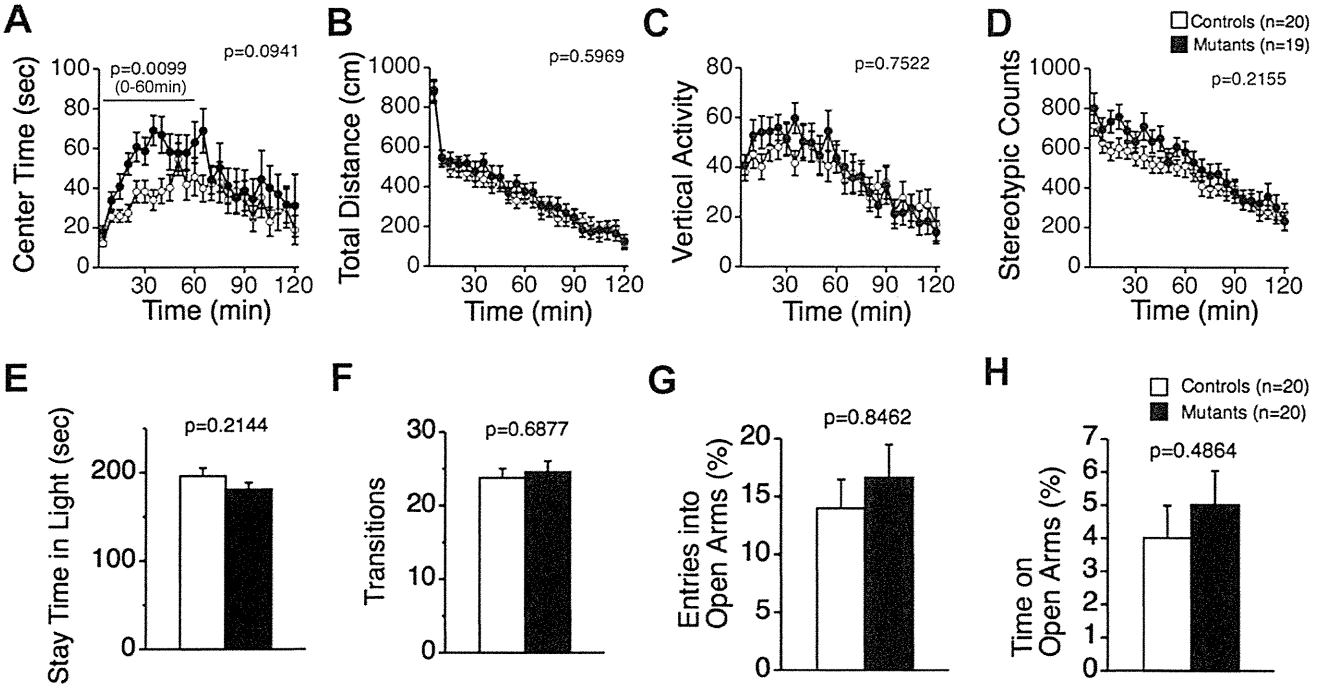

Figure 5. Normal locomotor activity in CD47 KO mice. In the open field test, (A) CD47 KO mice stayed significantly longer in the center of area of the apparatus than wild-type mice during the earlier part of the experimental period, but not over the entire experimental period. No significant differences were detected in total distance traveled (B), vertical activity (C), or stereotypic counts (D). CD47 KO mice, n = 19; wild-type mice, n = 20. CD47 KO mice and wild-type mice were also subjected to the light/dark transition test (E–F). Time spent in the light box (E) and number of transitions (F) did not significantly differ between genotypes. CD47 KO mice, n = 20; wild-type mice, n = 20. The elevated plus maze test was also performed (G–H). There were no significant differences between the genotypes in the number of entries to the open arms (G) or time spent on the open arms (H). CD47 KO mice, n = 20; wild-type mice, n = 20.
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The present study also failed to detect impairments of learning and memory in CD47 KO mice in the fear conditioning and Barnes maze tests. On the other hand, in an inhibitory avoidance learning paradigm, previous studies demonstrated that CD47 deficiency led to an impairment of learning and memory in CD47 KO mice and CD47 antisense-injected rats in an inhibitory avoidance learning paradigm (Table S1) [22,23]. There are several possible explanations for these inconsistent results. First, differences in species, genetic backgrounds, and age may cause differences in learning and memory performance. In the paper by Chang et al., the CD47-null allele was backcrossed from $CD47^{+/-}$ 129sv/eg mice into Balb/cJ [23], whereas in the present study the mutants tested were CD47 KO mice that were backcrossed to C57BL/6J. Huang et al. used rats in their study [22]. In addition, the mutants in the paper by Chang et al. were subjected to the inhibitory avoidance test at 14–21 weeks of age [23], while the Barnes maze and fear conditioning tests were performed in the present study using mice at 39–48 and 43–52 weeks of age, respectively. There is therefore the possibility that CD47 KO leads to deficits in learning and memory performance only in young adult mice. Second, it is also possible that experiences during the behavioral test battery carried out prior to the Barnes maze and fear conditioning tests caused the different genotype effects on learning and memory performance. Third, there are known differences between the mechanisms of fear memory consolidation as it occurs in assays of fear conditioning and inhibitory avoidance learning [28,29]. For instance, pharmacological inactivation of $GABA_A$ receptors in the lateral and basal nuclei of the amygdala indicate that the amygdala could play an essential role in the acquisition of fear conditioning, and contributes to the modulation of memory consolidation of inhibitory avoidance, but not of fear conditioning [28]. It is therefore possible that CD47 plays a role in regulation of amygdala-dependent learning and memory via the $GABA_A$ receptor-mediated signaling. Lastly, the passive avoidance test could yield results that are inconsistent with those in the fear conditioning test. For example, muscarinic acetylcholine receptor M2 KO mice in one study demonstrated no impairments in fear conditioning, despite the fact that M2 KO led to impairment in the acquisition of a passive avoidance task [30]. In another example, GSK-3α KO mice exhibited a deficit in fear conditioning, but their learning and memory performance in the passive

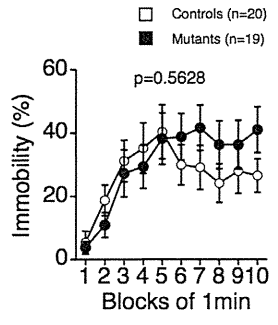

Figure 6. Normal depression-like behavior of CD47 KO mice in the tail suspension test. There was no significant genotype effect for the percentage of immobility time in the tail suspension test. CD47 KO mice, n = 19; wild-type mice, n = 20.
doi:10.1371/journal.pone.0089584.g006

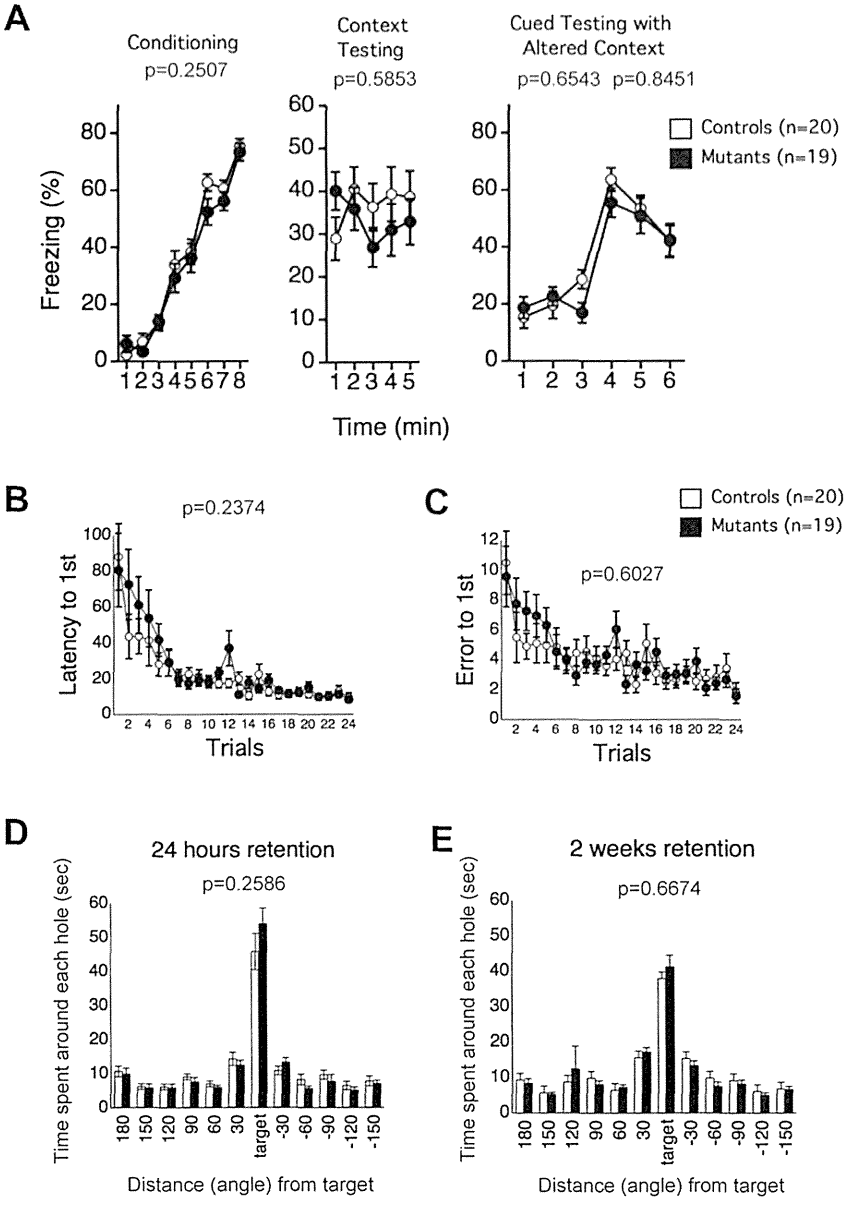

Figure 7. Normal fear and spatial memory in CD47 KO mice. (A) In the fear conditioning test, no significant differences between genotypes were observed in freezing during conditioning, context testing, or cued testing in an altered context. CD47 KO mice, n = 19; wild-type mice, n = 20. (B–E) In the training session of the Barnes maze test, no significant differences between the genotypes were detected in the latency to the 1st hole (the escape box) (B), or error to the 1st hole in the probe test (C). In a retention test performed either 24 h (D) or 2 weeks (E) after the last training session, there were no significant differences between the genotypes in time spent around the hole where the escape box had been placed (target). *CD47 KO mice, n = 19; wild-type mice, n = 20.*
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avoidance test was normal [31]. Additional studies, including histological and electrophysiological analyses, will be required to elucidate whether and how CD47 contributes to the regulation of learning and memory in mice. Using Hematoxylin & Eosin (HE) staining, we failed to detect obvious differences in the overall structure of the hippocampus between the genotypes (data not shown). In addition to the hippocampus, further precise analyses would be needed in the amygdala and other brain areas.

It is notable that a few studies reported that expression of CD47 is dysregulated in patients with psychiatric disorders including schizophrenia and attention deficit hyperactivity disorder (ADHD) [32–34]. In particular, the expression of CD47 is down-regulated in Brodmann area 46 in the prefrontal cortex [32], and up-regulated in whole blood of patients with schizophrenia [33]. In patients with ADHD, a rare copy number variation (CNV) was identified in the proximal 5′-upstream region of the CD47 gene [34]. The present study suggests that CD47 deficiency in mice leads to decreased PPI, and, potentially, abnormal social behavior, both of which are considered to be behavioral abnormalities relevant to schizophrenia [35]. Abnormal social behavior is also thought to be a trait related to ADHD [36]. On the other hand, this study failed to detect the impaired working memory in CD47 KO mice that is often observed in the patients with schizophrenia [35] and ADHD [36]. CD47 KO mice may thus recapitulate

selected aspects of schizophrenia and ADHD, suggesting that CD47 may represent a new therapeutic target for these conditions.

In conclusion, the present study suggests that CD47 is potentially involved in the regulation of sensorimotor gating, social behavior, and gait in mice. It remains unclear how the deficiency of CD47 leads to the behavioral phenotypes of the mutants. Further studies are required to determine the sites of the brain, developmental stages, and possible mechanisms that contribute importantly to produce the behavioral phenotypes of CD47 KO mice.

Methods

Ethical Statement

All animal care, behavioral testing procedure, and animal experiments were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (Permit No., MedKyo 09539) and the Institutional Animal Care and Use Committee of Fujita Health University (Permit No., I0741), based on the Law for the Humane Treatment and Management of Animals (2005) and the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (2006). Every effort was made to minimize the number of animals used.

Animals and Experimental Design

CD47 KO mice were generated as previously reported [37]. They were backcrossed onto a C57BL/6J line for at least ten generations. Mice were group housed (2–4 mice per cage) in a room with a 12-h light/dark cycle (lights on at 7:00 a.m.) with access to food and water ad libitum. Behavioral testing was performed between 9:00 a.m. and 6:00 p.m. except where otherwise indicated. Before testing each animal, each apparatus was cleaned with diluted sodium hypochlorite solution to prevent a bias due to olfactory cues. All behavioral tests (Table 1) were conducted in a manner similar to those previously described [38,39]. Prior to brain dissection, mice were deeply anesthetized with chloral hydrate (245 mg/kg, i.p.), and the brains were quickly removed. The raw data of behavioral tests, which are not described in this paper, are disclosed in the gene-brain-phenotyping database (http://www.mouse-phenotype.org/).

Hot Plate Test

The hot plate test was used to evaluate sensitivity to a painful stimulus. Mice were placed on a 55.0 (± 0.3)°C hot plate (Columbus Instruments, Columbus, OH), and latency to the first hind-paw response was recorded with a 15-sec cut-off time. The hind-paw response was defined as either a foot shake or a paw lick.

Motor Function Tests

A wire hang test apparatus (O'Hara & Co., Tokyo, Japan) was used to assess balance and grip strength. The apparatus consists of a box (21.5×22×23 cm) with a wire mesh grid (10×10 cm) on its top, which can be inverted. The mouse was placed on the wire mesh, which was then inverted, causing the animal to grip the wire. Latency to fall was recorded, with a 60-s cut-off time. A grip strength meter (O'Hara & Co.) was used to assess forelimb grip strength. Mice were lifted and held by their tail so that their forepaws could grasp a wire grid. The mice were then gently pulled backward by the tail with their posture parallel to the surface of the table until they release the grid. The peak force applied by the forelimbs of the mouse was recorded in newtons (N). Each mouse was tested three times and the highest value obtained was used for statistical analysis. Motor coordination and balance were tested with the rotarod test. The rotarod test, using an accelerating rotarod (UGO Basile Accelerating Rotarod, Varese, Italy), was performed by placing mice on rotating drums (3 cm diameter) and measuring the time each animal was able to maintain its balance on the rod. The speed of the rotarod accelerated from 4 to 40 rpm over a 5-min period.

Table 1. Comprehensive behavioral test battery of CD47 KO mice.

Test	Age (w)	Results
Forced Swim Test	12~18	[21]
General Health	12~21	Figure 1
Light/Dark Transition Test	12~21	Figure 5
Open Field Test	12~21	Figure 5
Social Interaction Test (Crawley's version)	13~22	Figure 3
Elevated Plus Maze Test	14~23	Figure 5
Hot Plate Test	14~23	Figure 1
Social Interaction Test (novel environment)	14~23	Figure 4
Rotarod Test	15~24	Figure 1
Startle Response/Prepulse Inhibition	16~25	Figure 2
Barnes Maze Test	39~48	Figure 7
Fear Conditioning Test	43~52	Figure 7
Gait Analysis Test	44~53	Figure 1
Tail Suspension Test	44~53	Figure 6
Social Interaction Test (home cage)	60~69	Figure 4

Age (w): age in weeks at the beginning of each test.
doi:10.1371/journal.pone.0089584.t001

Gait Analysis

We analyzed gait of adult mice during walk/trot locomotion by ventral plane videography as described [40] using DigiGait Imaging System (Mouse Specifics Inc, Watertown, MA). This system enables mice to walk on a motorized transparent treadmill belt, and the software automatically identifies the stance and swing components of stride, and calculates stance width, stride length, step angle, and paw angle. Briefly, we placed the mice on a treadmill belt that moves at a speed of 24.7 cm/s. We collected digital video images of the underside of mice at 150 frames per second.

Startle Response/prepulse Inhibition Tests

A startle reflex measurement system (O'Hara & Co.) was used to measure startle response and prepulse inhibition [39]. A test session began by placing a mouse in a Plexiglas cylinder where it was left undisturbed for 10 min. White noise (40 msec) was used as the startle stimulus for all trial types. The startle response was recorded for 140 msec (measuring the response every 1 msec) starting with the onset of the prepulse stimulus. The background noise level in each chamber was 70 dB. The peak startle amplitude recorded during the 140 msec sampling window was used as the dependent variable. A test session consisted of 6 trial types (2 types for startle stimulus only trials and 4 types for prepulse inhibition trials). The intensity of startle stimulus was 110 or 120 dB. The prepulse sound was presented 100 msec before the startle stimulus, and its intensity was 74 or 78 dB. Four combinations of prepulse and startle stimuli (74–110, 78–110, 74–120, and 78–120 dB) were employed. Six blocks of the 6 trial types were presented in pseudorandom order such that each trial type was presented once

within a block. The average inter-trial interval was 15 sec (range: 10–20 sec).

Crawley's Sociability and Preference for Social Novelty Test

The testing apparatus consisted of a rectangular, three-chambered box and a lid with an infrared video camera (O'Hara & Co.). Each chamber was 20×40×22 cm and the dividing walls were made from clear Plexiglas, with small square openings (5×3 cm) allowing access into each chamber. An unfamiliar C57BL/6 J male (stranger 1), that had had no prior contact with the subject mice, was placed in one of the side chambers. The location of stranger 1 in the left vs. right side chamber was systematically alternated between trials. The stranger mouse was enclosed in a small, round wire cage, which allowed nose contact between the bars, but prevented fighting. The cage was 11 cm in height, with a bottom diameter of 9 cm, vertical bars 0.5 cm apart. The subject mouse was first placed in the middle chamber and allowed to explore the entire test box for a 10-min session. The amount of time spent in each chamber was measured with the aid of a camera fitted on top of the box. Each mouse was tested in a 10-min session to quantify social preference for the first stranger. After the first 10-min session, a second unfamiliar mouse was placed in the chamber that had been empty during the first 10-min session. This second stranger was also enclosed in an identical small wire cage. The test mouse thus had a choice between the first, already-investigated unfamiliar mouse (stranger 1), and the novel unfamiliar mouse (stranger 2). The amount of time spent in each chamber during the second 10-min was measured as described above. Data acquisition and analysis were performed automatically using Image CSI software (see 'Data analysis').

Social Interaction Test in a Novel Environment

In the social interaction test, two mice of identical genotypes that were previously housed in different cages were placed in a box together (40×40×30 cm) and allowed to explore freely for 10 min. Social behavior was monitored with a CCD camera connected to a Macintosh computer. Analysis was performed automatically using Image SI software (see 'Data analysis'). The total number of contacts, total duration of active contacts, total contact duration, mean duration per contact, and total distance traveled were measured. The active contact was defined as follows. Images were captured at 1 frame per second, and distance traveled between two successive frames was calculated for each mouse. If the two mice contacted each other and the distance traveled by either mouse was longer than 4 cm, the behavior was considered as 'active contact'.

Social Interaction Test in Home Cage

Social interaction monitoring in the home cage was conducted as previously described [41]. The system was same apparatus in locomotor activity in home cage. Two mice of the same genotypes that had been housed separately were placed together in a home cage. Their social behavior was then monitored for 9 days. Output from the video camera was fed into a Macintosh computer. Images from each cage were captured at a rate of one frame per second. Social interaction was measured by counting the number of particles detected in each frame: two particles indicated that the mice were not in contact with each other; and one particle (i.e., the tracking software could not distinguish two separate bodies) indicated contact between the two mice. We also measured locomotor activity during these experiments by quantifying the number of pixels that changed between each pair of successive frames. Only eight pairs of mice were used for each genotype due to the limited availability of apparatus. Data acquisition failed in one apparatus, which was measuring the activity of a pair of KO mice. Therefore, we analyzed the data for the remaining eight pairs of WT and seven pairs of KO mice. Analysis was performed automatically using Image HA software (see 'Data analysis').

Open-field Test

Locomotor activity was measured using an open-field test as previously described [39]. Each mouse was placed in the corner of the open-field apparatus (40×40×30 cm; Accuscan Instruments, Columbus, OH). The test chamber was illuminated at 100 lux. Total distance traveled, vertical activity (rearing measured by counting the number of photobeam interruptions), time spent in the center area (20×20 cm), and beam-break counts for stereotypic behaviors were recorded. Data were collected for 120 min.

Light/dark Transition Test

The light/dark transition test was conducted as previously described [42,43]. The apparatus used for the light/dark transition test consisted of a cage (21×42×25 cm) divided into two sections of equal size by a partition with a door (O'Hara & Co.). One chamber was made of white plastic and brightly illuminated, whereas the other chamber was black and dark. Mice were placed in the dark side and allowed to move freely between the two chambers with the door open for 10 min. The number of transitions between the two compartments, latency to first enter the lit chamber, distance traveled, and time spent in each chamber were recorded by Image LD4 software (see 'Data analysis').

Elevated Plus-maze Test

The elevated plus-maze test was performed as previously described [44]. The apparatus consisted of two open arms (25×5 cm) and two enclosed arms of the same size, with 15-cm high transparent walls (O'Hara & Co.). The arms and central square were made of white plastic plates and were elevated to a height of 55 cm above the floor. To minimize the likelihood of animals falling from the apparatus, 3-mm high plastic ledges were provided for the open arms. Arms of the same type were arranged at opposite sides to each other. Each mouse was placed in the central square of the maze (5×5 cm), facing one of the closed arms. Mouse behavior was recorded during a 10-min test period. The number of entries into, and the time spent in open and enclosed arms, were recorded. For data analysis, we used the following four measures: the percentage of entries into the open arms, the time spent in the open arms (s), the number of total entries, and total distance traveled (cm). Data acquisition and analysis were performed automatically using Image EP software (see 'Data analysis').

Tail Suspension Test

The tail suspension test was performed for a 10-min test session. Mice were suspended 30 cm above the floor of a white plastic chamber (31×41×41 cm) (O'Hara & Co.) in a visually isolated area by adhesive tape placed ~1 cm from the tip of the tail, and the behavior was recorded over a 10-min test period. Images were captured at one frame per second. Immobility was judged by the application program according to a certain threshold. Immobility lasting for less than a 2 sec was not included in the analysis. Data acquisition and analysis were performed automatically, using Image TS software (see 'Data analysis').

Contextual and Cued Fear Conditioning Test

Each mouse was placed in a test chamber (26×34×29 cm) inside a sound-attenuated chamber (chamber A; O'Hara & Co.) and allowed to explore freely for 2 min. A 60-dB white noise, which served as the conditioned stimulus (CS), was presented for 30 s, followed by a mild (2 sec, 0.35 mA) footshock, which served as the unconditioned stimulus (US). Two more CS-US pairings were presented with a 2-min inter-stimulus interval. Context testing was conducted 24 hr after conditioning in the same chamber. Cued testing with altered context was conducted after conditioning using a triangular box (35×35×40 cm) made of white opaque Plexiglas, which was located in a different room. The chamber of the test was illuminated at 100 lux. In cued testing with altered context, white noise was delivered from 180 sec and continued until 6 min. Data acquisition, control of stimuli (tones and shocks), and data analysis were performed automatically using Image FZ software (see 'Data analysis'). Images were captured at 1 frame per second. For each pair of successive frames, the area in which the mouse moved was measured. When this area was below a threshold of 20 pixels, the behavior was judged as 'freezing', i.e., complete lack of mobility in any parts of the body during a 1-sec period. When the area equaled to or exceeded the threshold, the behavior was considered 'non-freezing'. The optimal threshold (amount of pixels) to judge freezing was determined by adjusting it to the amount of freezing measured by human observation. 'Freezing' that lasted less than the defined time threshold of 2 sec was not included in the analysis. The parameters were constant for all mice assessed.

Barnes Maze Test

The Barnes maze test was conducted on 'dry land', a white circular surface, 1.0 m in diameter, with 12 holes equally spaced around the perimeter (O'Hara & Co.). The circular open field was elevated 75 cm from the floor. A black Plexiglas escape box (17×13×7 cm) containing paper cage bedding on its floor was located under one of the holes. The hole above the escape box represented the target, analogous to the hidden platform in the Morris task. The location of the target was consistent for a given mouse but was randomized across mice. The maze was rotated daily, with the spatial location of the target unchanged with respect to the visual room cues, in order to prevent bias based on olfactory or proximal cues within the maze. One trial per day for 7 successive days and two trials per day for next 6 successive days were conducted except for no trial on day 6 and one trial on the last day. A probe trial was conducted 24 hr after the last training session without the escape box in order to confirm that this spatial task was performed based on navigation using distal environment room cues. Time of latency to reach the target hole, number of errors, distance to reach the target hole, and time spent around each hole were recorded by Image BM software (see 'Data analysis'). To assess long-term retention, a second probe trial was applied a week after probe test 1 and additional one session of retraining.

Data Analysis

The applications used for the behavioral studies (Image LD4, Image SI, Image OF, Image TS, Image SI, Image HA, Image CSI, Image FZ, Image BM) were developed by Dr. Tsuyoshi Miyakawa (available through O'Hara & Co.) based on NIH Image program (NIH, Bethesda, MD, available at http://rsb.info.nih.gov/nih-image/) and ImageJ (ImageJDev.Org, available at http://imagejdev.org/). Statistical analysis was conducted using StatView (SAS Institute, Cary, NC). Data were analyzed using a paired *t*-test, Student's *t*-test, Mann-Whitney U-test, one-way ANOVA, or two-way repeated measures ANOVA. Values in graphs are expressed as mean ± SEM.

Supporting Information

Table S1 Behavioral phenotypes in CD47 KO and SIRP-alpha mutant mice.
(XLSX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: HO T. Miyakawa. Performed the experiments: KT HO. Analyzed the data: HK KT HO T. Miyakawa. Contributed reagents/materials/analysis tools: T. Matozaki HO T. Miyakawa. Wrote the paper: HK KT T. Miyakawa.

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JB Review

The CD47–SIRPα signalling system: its physiological roles and therapeutic application

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Signal regulatory protein α (SIRPα), also known as SHPS-1/BIT/ CD172a, is an immunoglobulin superfamily protein that binds to the protein tyrosine phosphatases SHP-1 and SHP-2 through its cytoplasmic region. CD47, another immunoglobulin superfamily protein, is a ligand for SIRPα, with the two proteins constituting a cell–cell communication system (the CD47–SIRPα signalling system). SIRPα is particularly abundant in the myeloid-lineage hematopoietic cells such as macrophages or dendritic cells (DCs), whereas CD47 is expressed ubiquitously. Interaction of CD47 (on red blood cells) with SIRPα (on macrophages) is thought to prevent the phagocytosis by the latter cells of the former cells, determining the lifespan of red blood cells. Recent studies further indicate that this signalling system plays important roles in engraftment of hematopoietic stem cells as well as in tumour immune surveillance through regulation of the phagocytic activity of macrophages. In the immune system, the CD47–SIRPα interaction is also important for the development of a subset of CD11c$^+$DCs as well as organization of secondary lymphoid organs. Finally, the CD47–SIRPα signalling system likely regulates bone homeostasis by osteoclast development. Newly emerged functions of the CD47–SIRPα signalling system thus provide multiple therapeutic strategies for cancer, autoimmune diseases and bone disorders.

Keywords: cancer therapy/CD47–SIRPα system/dendritic cell/macrophage/phagocytosis.

Abbreviations: BIT, brain immunoglobulin-like molecule with tyrosine-based activation motifs; CCL, chemokine (C-C motif) ligand; cDC, conventional dendritic cell; CHS, contact hypersensitivity; DCs, dendritic cells; EAE, experimental autoimmune encephalomyelitis; FRC, fibroblastic reticular cells; HSCs, hematopoietic stem cells; IFN, interferon; Ig, immunoglobulin; IL, interleukin; M-CSF, macrophage colony-stimulating factor; NK, natural killer; NOD, non-obese diabetic; RANKL, receptor activator of nuclear factor-κB ligand; RBCs, red blood cells; SH2, Src homology region 2; SHP-1, Src homology region 2 domain-containing phosphatase-1; SHP-2, Src homology region 2 domain-containing phosphatase-2; SHPS-1, Src homology 2 domain-containing protein tyrosine phosphatase substrate-1; SIRPα, signal regulatory proteinα; SLOs, secondary lymphoid organs; Th, T-helper.

Signal regulatory protein α (SIRPα) [also known as Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1), brain immunoglobulin (Ig)-like molecule with tyrosine-based activation motifs (BIT) or CD172a] is a transmembrane protein that contains three Ig-like domains in the extracellular region and four tyrosine residues for putative phosphorylation in the cytoplasmic region (*1–3*) (Fig. 1). The tyrosine phosphorylation sites of SIRPα bind and thereby activate Src homology region 2 (SH2) domain-containing phosphatase-1 (SHP-1) or SHP-2 through their SH2 domains (Fig. 1). SIRPα was originally identified as a highly expressed glycoprotein in the brain as well as a binding partner or a putative substrate for SHP-1 and SHP-2 (*4–7*). Tyrosine phosphorylation of the cytoplasmic region of SIRPα is triggered by various growth factors and cytokines as well as by integrin-mediated cell adhesion to extracellular matrix proteins (*5*, *8*). SHP-1 is predominantly expressed in hematopoietic cells and negatively regulates various functions of these cells (*9*). In contrast, SHP-2 is expressed in most cell types and regulates the small guanosine triphosphate (GTP)-binding proteins Ras and Rho, thereby contributing to the positive control of cell growth and cell migration, respectively (*6*, *10*, *11*). SIRPα thus functions as a docking protein to recruit and activate SHP-1 or SHP-2 at the cell membrane in response to extracellular stimuli, and these protein phosphatases are thought to be important for signalling downstream of SIRPα. SIRPα is especially abundant in neurons as well as in myeloid-lineage hematopoietic cells such as macrophages or dendritic cells (DCs) (*3*, *12–14*).

The extracellular region of SIRPα interacts with its ligand, CD47, through an IgV-like domain at the NH_2-terminus of the extracellular region of SIRPα. CD47, which was originally identified in association with $\alpha_v\beta_3$

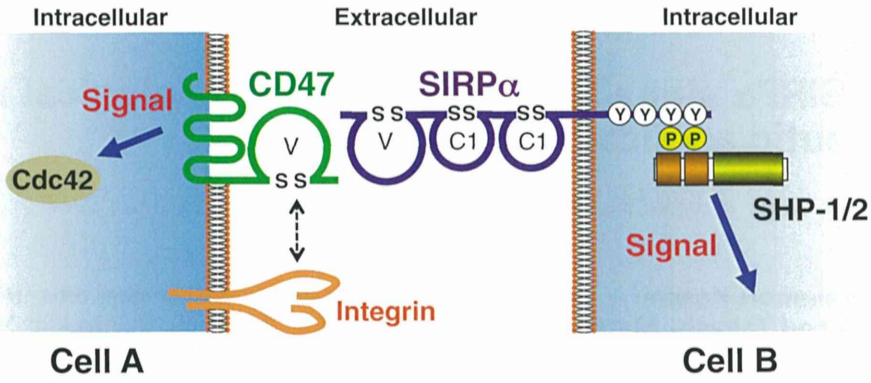


Fig. 1 The CD47–SIRPα signalling system. SIRPα is a transmembrane protein that contains three Ig-like domains (one V-like and two C1-like Ig domains) in its extracellular region and two tyrosine phosphorylation sites in its C-terminal cytoplasmic region. The tyrosine-phosphorylated sites of SIRPα bind to the protein tyrosine phosphatases, SHP-1 and SHP-2 and thereby activate these phosphatases. SIRPα ligand, CD47 is also a member of the Ig superfamily, possessing a V-type Ig-like extracellular domain, five membrane-spanning segments and a short cytoplasmic tail. The N-terminal IgV-like domain of SIRPα *trans*-interacts with the IgV-like domain of CD47. The binding of CD47 to SIRPα promotes the tyrosine phosphorylation of the latter protein. This binding also activates Cdc42, a Rho family small GTP-binding protein, downstream of CD47. In addition, CD47 *cis*-interacts with integrins through its extracellular region and thereby regulates their function.

integrin, is also a member of the Ig superfamily of proteins, possessing an IgV-like extracellular domain, five putative membrane-spanning segments and a short cytoplasmic tail (Fig. 1) (*15*, *16*). In contrast to the relatively restricted expression of SIRPα, CD47 is expressed in most cell types. Ligation of SIRPα by CD47 promotes tyrosine phosphorylation of the cytoplasmic region of the former protein in macrophages (*14*, *17*). In the cytoplasmic region of SIRPα, there are four putative tyrosine phosphorylation sites, and the C-terminal two tyrosine phosphorylation sites likely provide the binding sites for SHP-1 or SHP-2 (*18*) (Fig. 1). These protein tyrosine phosphatases appear to mediate the functions of SIRPα through dephosphorylating their substrates. In contrast, the ligation of CD47 by SIRPα promotes activation of Cdc42, a member of the Rho family of small GTP-binding proteins, in neurons (*19*), although the identity of the molecular components conducting signalling downstream of CD47 remains largely unclear (Fig. 1). Regarding the functions of CD47, particularly through activation of integrin, or the molecular mechanism for its actions, audience of this article can refer to other excellent reviews published elsewhere (*15*, *16*).

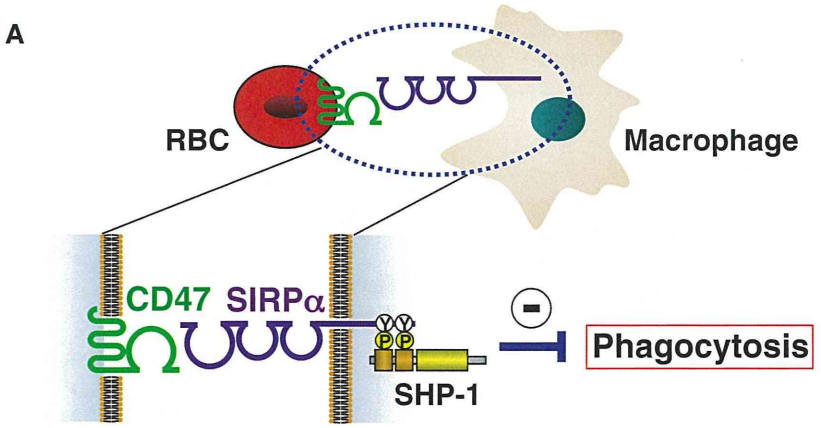
The mechanism by which the complex of CD47 and SIRPα is removed from the cell surface has remained unknown. Ectodomain shedding of SIRPα could be one mechanism for such removal. However, it unlikely occurs after the binding of CD47 to SIRPα (*20*). In contrast, co-culture of CD47-expressing CHO cells and SIRPα-expressing CHO cells results in endocytosis of the ligand–receptor complex into either cell type (*trans*-endocytosis) (*21*). Moreover, CD47 expressed on the surface of cultured mouse hippocampal neurons undergoes *trans*-endocytosis by neighbouring astrocytes expressing endogenous SIRPα (*21*). Thus, *trans*-endocytosis of CD47 likely acts as a mechanism by which the complex of CD47 and SIRPα is removed from the cell surface in order to terminate the cell responses induced by the CD47–SIRPα interaction.

Regulation of Phagocytosis of Macrophages

SIRPα is predominantly expressed in phagocytes such as macrophages, 'professional' phagocytes, which play an important role in preservation of tissue integrity and function by engulfing old cells or apoptotic bodies (*22*, *23*). The CD47–SIRPα signalling system has been shown to play crucial roles in regulation of phagocytosis by macrophages of matured blood cells such as red blood cells (RBCs) in the spleen. Recent studies further demonstrate that this signalling system plays important roles in the rejection of transplanted hematopoietic stem cells (HSCs) and in tumour immune surveillance through regulation of the phagocytic activity of macrophages.

Phagocytosis of matured RBCs

The role of CD47–SIRPα signalling system in the prevention of phagocytosis of matured blood cells (Fig. 2A) was first indicated by Oldenborg *et al.* (*17*, *24*); They showed that the rate of clearance of transfused CD47-deficient RBCs from the bloodstream of wild-type mice was markedly increased compared with that apparent for transfused wild-type cells (*17*, *24*). It was also shown that the phagocytosis of CD47-deficient RBCs by isolated splenic macrophages from wild-type mice was greatly enhanced *in vitro* (*17*). In addition, the phagocytic response was enhanced in 'motheaten viable' mice, in which the activity of SHP-1 was genetically defective, compared with that in wild-type mice (*24*), suggesting that SHP-1 is implicated in such regulation (Fig. 2A). Subsequently, the rate of clearance of transfused wild-type RBCs from the bloodstream was markedly increased in SIRPα mutant mice, which express a mutant form of SIRPα that lacks most of the cytoplasmic region and thus is unable to bind SHP-1 (*25*). Phagocytosis of opsonized RBCs by isolated macrophages from these SIRPα mutant mice was also enhanced (*14*). Collectively, these observations indicated that the binding of CD47 on RBCs to SIRPα on splenic macrophages prevents phagocytosis of the RBCs by the macrophages,



B

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                29                                                                  88
NOD             ATRTEVKVIQPEKSVSVAAGDSTVLNCTLTSLLPVGPIRWYRGVGQSRQLIYSFTTEHFP
C57BL/6         A.GK.L..T.............V....L.........R......P..L.....AG.YV.
BALB/c          A.GT.V..T.............I....V.........R......Q..L.....TG.HF.
129             V.GK.L..T.............V....L.........K......Q..L.....TG.HF.

                89                                                                 147
NOD             RVTNVSDATKRSNLDFSIRISNVTPEDAGTYYCVKFQRGS--PDTEIQSGGGTEVYVLA
C57BL/6         .IR....T...N.M...........A...I.......K.SSE.................
BALB/c          .VR....T...N.M...........E...T.......R.SSE.................
129             .VT....A...N.M...........E...T.......R.PSE.................
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C

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                27                                                                  86
EnsEMBL_V1      GVAGEEELQVIQPDKSVLVAAGETATLRCTATSLIPVGPIQWFRGAGPGRELIYNQKEGH
NA06994_V2      G.....E........S.S.....S.I.H..V.................A............
NA07056_V3      G.....E........S.S.....S.I.L..V.................A............
NA11832_V4      G.....G........S.S.....S.I.H..A.................G............
NA18502_V5      G.....E........F.L.....T.T.R..A.................G............
NA18507_V6      G.....E........S.L.....T.T.R..A.................G............
NA18524_V7      G.....E........S.S.....S.I.H..V.................A............
NA18570_V8      G.....E........S.L.....T.T.R..A .................A............
NA18943_V9      G.....E........S.L.....T.T.R..A.................G............
NA18949_V10     R.....E........S.S.....S.I.H..V.................A............

                87                                                                 146
EnsEMBL_V1      FPRVTTVSDLTKRNNMDFSIRIGNITPADAGTYYCVKFRKGSPDDVEFKSGAGTELSVRA
NA06994_V2      ........ES...E....S.S.S.....................-.T.............A
NA07056_V3      ........ES...E....S.S.S.....................-.T.............A
NA11832_V4      ........DL...N....S.R.G.....................V..............A
NA18502_V5      ........DL...N....S.R.G.....................V..............A
NA18507_V6      ........DL...N....P.R.G.....................V..............A
NA18524_V7      ........ES...E....S.S.S.....................-.T.............G
NA18570_V8      ........ES...E....S.S.S.....................-.T.............A
NA18943_V9      ........DL...N....S.R.S.....................V..............A
NA18949_V10     ........ES...E....S.S.S.....................-.T.............A
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Fig. 2 Regulation of phagocytosis of macrophage by the CD47–SIRPα singling system. (A) Ligation of SIRPα (on macrophages) by CD47 (on RBCs) promotes tyrosine phosphorylation of SIRPα and its subsequent association with the phosphatase SHP-1, resulting in inhibition of phagocytosis of RBCs by macrophages. (B, C) Alignments of the amino acid sequence of the N-terminal IgV-like domain of NOD SIRPα (aa. 29–145) together with that of other mouse strains (B) described in other studies (*31*, *32*) and that of the N-terminal IgV-like domain of human SIRPα (EnsEMBL_v1) (aa. 27–146) with other polymorphic variants of human SIRPα (C) described in another study (*31*). Polymorphic residues that are different from those of NOD SIRPα (B) or from human SIRPα (C) (EnsEMBL_v1) are shown in Red. The amino acid residues of the N-terminal IgV-like domain of human SIRPα that are thought to be important for its binding to CD47 (*33*) are shown in Green (C).

thereby determining both the lifespan of individual RBCs and their number in the circulation (Fig. 2A). Moreover, it was subsequently shown that the binding of SIRPα on human monocytes to CD47 on RBCs negatively regulates the FcγR-dependent phagocytosis by dephosphorylation of myosin-IIA, a key molecule of phagocytosis, at phagocytic synapses (*26*). In contrast, it has been suggested that CD47 acts as a molecular switch for the regulation of aged RBC phagocytosis. In fact, a subset of old human RBCs in whole blood was found to undergo phagocytosis by human red pulp macrophages through the interaction of CD47 on the former cells with SIRPα on the latter cells *in vitro* (*27*). Such phagocytosis is dependent on a conformational change in CD47 on the RBCs, which appears to be induced by oxidation. However, the molecular mechanism by which SIRPα, through its binding to CD47, mediates induction of

phagocytosis of aged RBCs remains unclear. The CD47–SIRPα interaction is also implicated in prevention of the clearance by splenic macrophages of circulating platelets or lymphocytes (*28–30*).

Phagocytosis of transplanted HSCs

Graft failure in the transplantation of HSCs occurs despite donor–host genetic identity of human leucocyte antigens, suggesting that additional factors modulate engraftment. With the non-obese diabetic (NOD)-severe combined immunodeficiency (SCID) xenotransplantation model, it was demonstrated that the NOD background allows better engraftment of human HSCs than do other strains with equivalent immunodeficiency-related mutations (*31*). Moreover, expression of NOD SIRPα on macrophages was shown to be required for support of human hematopoiesis (*31*). The mouse *Sirpα* gene exhibits considerable polymorphism in the cording region and the amino acid sequence of SIRPα in the N-terminal IgV-like domain is particularly polymorphic between mouse strains (*31*, *32*) (Fig. 2B). In general, CD47 from one species is thought to barely interact with SIRPα from another (*34*, *35*), whereas the SIRPα polymorphic variant of NOD mice was found to have relatively high affinity for the human CD47 compared with that of other mouse strains, such as C57BL/6 and BALB/c mice (*31*, *36*). Thus, interaction of donor CD47 (on human HSCs) with recipient SIRPα (on macrophages) allows efficient engraftment of HSCs. In addition, while *Rag2*^{null}*Il2rg*^{null} mice on a C57BL/6 background, which lack T cells, B cells and natural killer (NK) cells, are unable to reconstitute human hematopoiesis the replacement of *Sirpα* gene in the mice by NOD *Sirpα* gene markedly enhanced human HSC engraftment in comparison with control mice (*37*). The forced expression of mouse CD47 in human HSCs also improved their engraftment into *Rag2*^{null}*Il2rg*^{null} mice on a BALB/c background (*38*). These findings further support the notion that the *trans*-interaction of CD47 on HSCs with SIRPα on macrophages is a key determinant of efficient HSC engraftment.

Of note is that 10 different sequence patterns exist in the N-terminal IgV domain of human SIRPα (Fig. 2C), and such SIRPα polymorphisms were proposed to be a potent genetic determinant of the engraftment of human HSCs (*31*). Co-crystal structure analysis of the N-terminal IgV-like domain of SIRPα, as well as of CD47, suggested that the polymorphic residues in human SIRPα are indeed not important for the binding of CD47 to SIRPα (*33*). However, the recent study also demonstrated that each polymorphic variant of human SIRPα possesses different affinity for human CD47 *in vitro* (*39*), suggesting the importance of the human SIRPα polymorphisms for success in HSCs transplantation.

Phagocytosis of tumour cells

Interaction of CD47 on tumour cells with SIRPα on the macrophages has recently been implicated to prevent the elimination by the latter cells of the former cells (Fig. 3). Furthermore, the disruption of such CD47–SIRPα interaction is proposed to be a new strategy for cancer

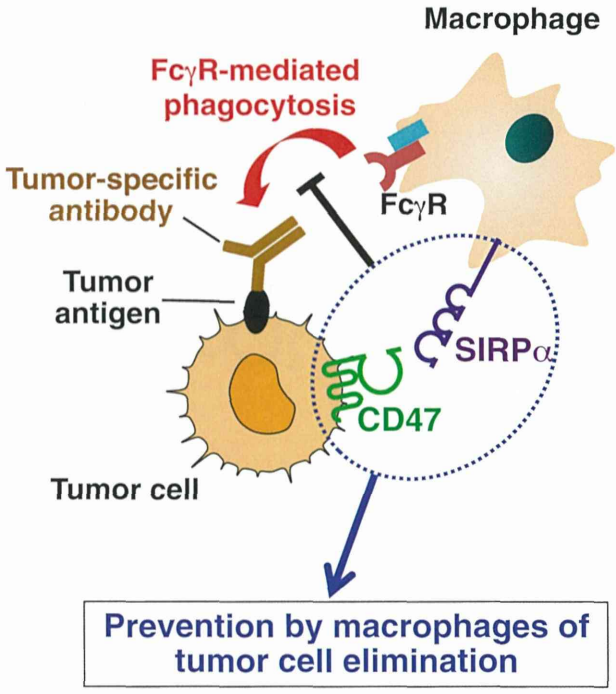

Fig. 3 Role of the CD47–SIRPα interaction in tumour immune surveillance. Interaction of SIRPα (on macrophages) with CD47 (on tumour cells) prevents the elimination by macrophages of tumour cells. The blockage of the CD47–SIRPα interaction in combination with therapeutic monoclonal antibodies specific for tumour antigens is a new strategy for cancer therapy.

treatment. Indeed, CD47 is identical to a well-known tumour antigen, OV-3, the level of which was markedly upregulated on ovarian carcinoma cells (*16*). It has recently been demonstrated that the expression level of CD47 is markedly increased in acute myeloid leukaemia stem cells (*40*), as well as B cell lymphoma (*41*) and solid tumour cells (*42*) compared with that apparent in normal counterparts. Elevated expression of CD47 was also shown to be associated with poor prognosis in patients with these malignancies (*40–43*). Forced expression of mouse CD47 in human myeloid leukaemia cells, which express endogenous CD47 at very low level and are unable to be engrafted in *Rag2*^{null}*Il2rg*^{null} mice, prevented phagocytosis of the tumour cells by macrophages and facilitated engraftment of the tumour cells in the mice (*44*). It was therefore proposed that the increased expression of CD47 on tumour cells allows for the evasion of phagocytosis by macrophages, resulting in promotion of tumour growth and dissemination *in vivo*. Moreover, it is suggested that targeting CD47–SIRPα pathway could be a promising therapeutic strategy for various human cancers. Indeed, the anti-CD47 antibody alone (*40*, *42*, *43*) or in combination with monoclonal antibodies to tumour antigens (*41*) was shown to reduce the growth of human leukaemia or solid tumour cells in mouse xenotransplantation models.

We have recently shown that SIRPα mutant mice bearing syngeneic melanoma cells, in which CD47 are highly expressed, exhibited a tumour formation similar to that for wild-type mice (*45*), indicating that SIRPα is not required for efficient engraftment and outgrowth of the tumour cells in mice. In contrast,

the treatment with therapeutic antibodies specific for a melanoma antigen was found to markedly reduce tumour burden in SIRPα mutant mice compared with that in wild-type mice (*45*), consistent with the notion that interaction of CD47 with SIRPα prevents the elimination by macrophages of cancer cells. Quite recently, the engineered SIRPα variants, which possess high affinity for CD47, are demonstrated to significantly enhance the efficacy of therapeutic antibodies for cancers, such as rituximab and alemtuzumab, in mouse xenograft models (*46*). Collectively, the blockage of the CD47–SIRPα interaction in combination with therapeutic monoclonal antibodies specific for tumour antigens is quite a promising strategy for cancer therapy (Fig. 3).

Roles of the CD47–SIRPα Signalling System in the Immune System

In terms of the expression in the immune cells, SIRPα is expressed in DCs as well as macrophages and neutrophils, whereas it is barely expressed in T cells, B cells, NK cells and NK T cells (*3*, *47*, *48*). In contrast, CD47 is expressed in all types of immune cells. Recently, the CD47–SIRPα interaction is shown to play important roles for development of DC in the secondary lymphoid organs (SLOs) as well as regulation of autoimmune diseases.

Homeostasis of DCs as well as of T cells in the SLOs

DCs are professional antigen-presenting cells, which are important for initiation of the immune responses of T cells to exogenous pathogens as well as maintenance of T cell tolerance to self-components. The mouse spleen harbours two major subtypes of DCs, namely $CD11c^{high}$ conventional DCs (cDCs) and plasmacytoid DCs, defined as $CD11c^{int}$ $B220^+$ cells. The latter DCs produce type I interferons in response to viral and bacterial pathogens. The cDCs are further classified into $CD4^+$ $CD8^-$ cDCs ($CD4^+$ cDCs), $CD4^-$ $CD8^-$ cDCs (DN cDCs) and $CD4^-$ $CD8^+$ cDCs ($CD8^+$ cDCs). $CD8^-$ cDCs are thought to be important for priming and development of $CD4^+$ helper T (Th) cells, whereas $CD8^+$ cDCs are for induction of cytotoxic $CD8^+$ T cells. Genetic analysis of cDC development and homeostasis revealed that many gene products regulate cDC development, with some of them selectively controlling $CD8^-$ cDCs or $CD8^+$ cDCs (*49*). The level of expression of SIRPα on $CD4^+$ cDCs and DN cDCs in the spleen is much greater than that on the corresponding $CD8^+$ cDCs (*48*, *50*, *51*). Indeed, SIRPα mutant mice manifest marked reduction in the number of splenic $CD8^-$ cDCs, in particular $CD4^+$ cDCs, as well as marked shorten in the half-life of $CD4^+$ cDCs in the spleen compared with those of wild-type mice, indicating that SIRPα is important for the survival of $CD4^+$ cDCs in the spleen (*51*). Similar to SIRPα mutant mice, CD47-deficent mice manifest the reduction of $CD8^-$ cDCs (*50–52*), suggesting the importance of the CD47–SIRPα interaction in the regulation of DC homeostasis.

Studies with bone marrow chimera mice revealed that SIRPα within cDCs or DC precursors is likely important for development of splenic $CD4^+$ cDCs (*51*). In contrast, both hematopoietic and nonhematopoietic CD47 was found to be required for homeostasis of splenic $CD4^+$ cDCs as well as DN cDCs. CD47 is expressed in splenic and bone marrow stromal cells (*53*) as well as in endothelial cells (*16*). Thus, *trans*-interaction of CD47 on either nonhematopoietic cells (such as stromal or endothelial cells) or hematopoietic cells (such as T or B cells) with SIRPα on DCs is likely to be involved in the homeostatic regulation of $CD8^-$ cDCs in the spleen (Fig. 4A).

Interaction of CD47 with SIRPα is also thought to play a role for T cell homeostasis in the spleen. The size of T cell zone and the number of resident $CD4^+$ T cells in the spleen are significantly reduced in SIRPα mutant mice (*54*). In addition, the expression of chemokine (C-C motif) ligand (CCL) 19, CCL21 or interleukin (IL)-7 is reduced in the spleen of SIRPα mutant mice (Fig. 4B). CD47-deficient mice also manifested these phenotypes similar to SIRPα mutant mice. Both CCL19 and CCL21 as well as IL-7 are produced by stromal cells, named fibroblastic reticular cells (FRC), in the spleen (*55*). CCL19 and CCL21 are thought to attract naïve T cells into periarterial lymphoid sheathes in the spleen. IL-7 is also thought to be important for organization and maintenance of T cell zone as well as for lymphoid tissue inducer cells, which participate in development of SLOs (*56*, *57*). Indeed, the expression of the gp38, which is predominantly expressed in FRCs, was decreased in the spleen of SIRPα mutant mice (*54*), suggesting that the decrease of cell population of FRCs is likely to be a cause for the reduced expression of CCL19, CCL21 or IL-7 in the spleen of SIRPα mutant mice. Moreover, signalling by lymphotoxin, which is important for development of SLOs (*56*, *58*), was also shown to be impaired in the spleen of SIRPα mutant mice (*54*). By the use of bone marrow chimera, hematopoietic SIRPα is shown to be important for development of T cell zone as well as the expression of CCL19 and CCL21 in the spleen (*54*). Collectively, interaction of CD47 with SIRPα (presumably on DCs or macrophages) is thus essential for steady-state homeostasis of T cells, likely by regulation of stromal cells, in the spleen (Fig. 4C).

Development of autoimmune diseases

Studies with SIRPα mutant or CD47-deficeint mice suggested that SIRPα and CD47 are important for the development of Th17 cell- or Th1 cell-related autoimmune diseases. SIRPα mutant mice were resistant to the development of myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (EAE), to collagen-induced arthritis and to 2,4-dinitro-1-fluorobenzene-induced contact hypersensitivity (CHS) as well as to IL-10 deficiency-induced colitis (*59–63*). The production of IL-17 or interferon (IFN)-γ by T cells from SIRPα mutant mice exposed to the antigens responsible for the induction of these conditions was found to be markedly impaired (*59*, *60*, *62*). In addition, CD47-deficent mice were also shown to be resistant to *in vivo* models for EAE, CHS and

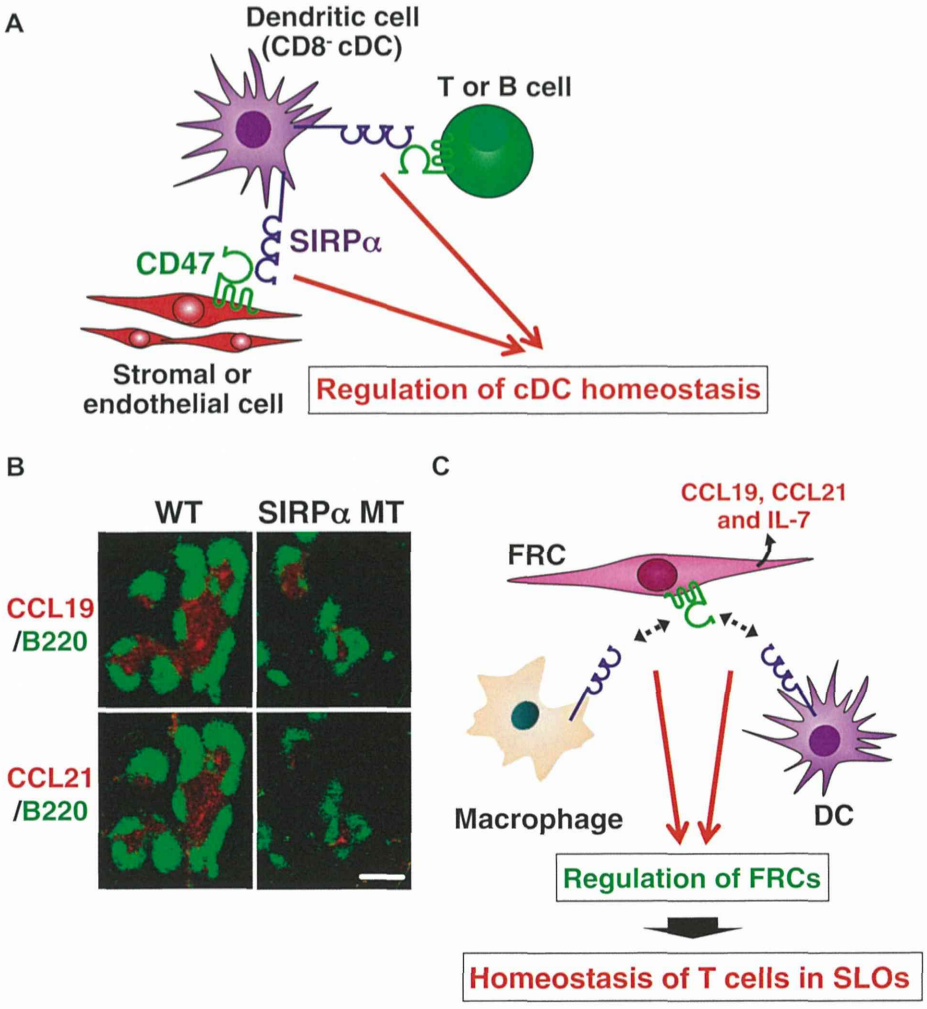

Fig. 4 Homeostasis of DCs as well as of T cells in the spleen. (A) Regulation by the CD47–SIRPα interaction of cDC homeostasis in the spleen. Interaction of SIRPα on $CD8^-$ cDCs with CD47 on nonhematopoietic cells (such as stromal or endothelial cells) or on hematopoietic cells (such as T or B cells) is likely to play an important role in the development and survival of $CD8^-$ cDCs in the spleen. (B) Reduced expression of chemokine CCL19 (Red; upper panels) or CCL21 (Red: lower panels) in the spleen of SIRPα mutant (MT) mice. Staining of B220 (B cell marker) is indicated by Green. WT, wild-type. Scale bar: 100 μm. (C) Homeostatic regulation by the CD47–SIRPα interaction of T cells in the spleen. The interaction of SIRPα (on either dendritic cells or macrophages) with CD47 (on FRCs) in the spleen likely participates in regulation of FRCs that produce CCL19, CCL21 and IL-7, thereby maintaining T cells homeostasis.

experimental colitis (*64–67*). Given that SIRPα expression is prominent in DCs, the function of DCs from SIRPα mutant or CD47-deficeint mice is likely impaired, resulting in a defect in the induction of Th1 or Th17 cells for the development of autoimmunity. However, the functions of DCs, such as maturation (*48*, *59*), migration (*68*) or proinflammatory cytokine production as well as induction of Th17 cells (Saito and Matozaki, unpublished data), in the SIRPα mutant mice were equivalent to that apparent with the wild-type mice. Thus, the marked reduction of cDCs as well as disorganization of SLOs are likely the causes of impaired development of autoimmunity in both SIRPα mutant and CD47-deficient mice.

Roles of CD47 and SIRPα in Bone Homeostasis

Osteoclasts mediate bone resorption and play important roles in normal bone development and regeneration. These cells are multinucleated cells derived from the monocyte-macrophage lineage and osteoclast development is coordinated with mesenchymal cells in bone, such as bone marrow stromal cells and osteoblasts. The first implication of CD47 and SIRPα in osteoclastogenesis and bone homeostasis was obtained from the studies, in which fusion and multinucleation of alveolar macrophages were prevented by disruption of the CD47–SIRPα interaction *in vitro* (*69*, *70*). Subsequent studies showed that CD47 and SIRPα are indeed expressed in osteoclasts and bone marrow stromal cells (*71*, *72*). The generation of osteoclasts was markedly impaired by depletion of CD47 or disruption of CD47–SIRPα interaction with their blocking antibodies in mouse bone marrow cell culture *in vitro* (*72–75*). In particular, interaction of CD47 and SIRPα within bone marrow stromal cells is, in part, important for osteoclast formation *in vitro* (*72*). CD47-deficient bone marrow culture *in vitro* also showed defects in osteoblastic differentiation and in

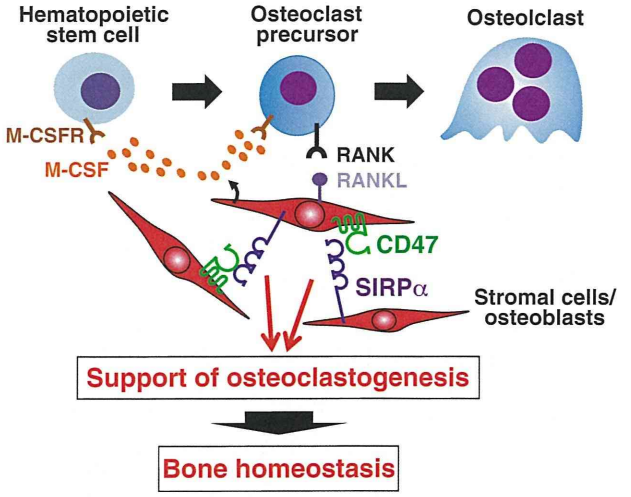

Fig. 5 Roles of CD47 and SIRPα in osteoclastogenesis. Osteoclast precursors differentiated from hematopoietic stem cells fuse with other precursors to form osteoclasts, giant multinucleated cells. Stromal cells and osteoblasts express M-CSF and receptor activator of nuclear factor-κB ligand (RANKL), both of which play a pivotal role in osteoclastogenesis by activation of their receptors on hematopoietic stem cells and osteoclast precursors. Interaction of SIRPα with CD47 within stromal cells/osteoblasts likely supports osteoclastogenesis and thereby contributes to bone homeostasis. M-CSFR, M-CSF receptor; RANK, receptor activator of nuclear factor-κB.

the production of macrophage colony-stimulating factor (M-CSF) as well as receptor activator of nuclear factor κB ligand (RANKL), both of which are important for osteoclastogenesis (*72*). Trabecular bone volume in CD47-deficient mice was markedly reduced compared with that for wild-type mice, with the numbers of osteoclasts and osteoblasts being markedly decreased (*72*). In contrast, Uluçkan *et al.* described that trabecular bone volume was rather increased in CD47-deficient mice and that tumour burden and bone loss were decreased in an intracardiac metastatic model (*75*). Although the results obtained with CD47-deficient mice are controversial, interaction of CD47 with SIRPα are likely important for osteoclastogenesis as well as bone homeostasis (Fig. 5).

In contrast to CD47-deficient mice, the development of and number of osteoclasts were comparable between SIRPα mutant and wild-type mice (*71*), whereas SIRPα mutant mice exhibited marked reduction of cortical bone volume. In addition, bone resorption by osteoclasts from SIRPα mutant mice was markedly enhanced *in vitro*, indicating that SIRPα plays an inhibitory role in the regulation of osteoclast activity (*71*). It remains unclear why the phenotypes of SIRPα mutant mice were different from that of CD47-deficent mice. However, the phenotypes of the former mice are likely independent of CD47–SIRPα interaction.

Concluding Remarks

Recent studies have clarified that the CD47–SIRPα signalling system plays important roles in regulation of phagocytosis, DC and T cell homeostasis and autoimmunity as well as bone homeostasis. Targeting CD47 and SIRPα might provide a new therapeutic strategy for hematopoietic disorders, autoimmune diseases and bone disorders as well as cancers. However, the molecular basis of CD47–SIRPα signalling system in regulation of DC and T cell homeostasis and autoimmunity as well as osteoclastogenesis remains not fully understood. For instance, the signalling pathway downstream of either SIRPα or of CD47 for regulation of DC and T cell homeostasis as well as osteoclastogenesis should be investigated further in future. As the use of reagents (such as antibodies to CD47 or SIRPα) that prevent the CD47–SIRPα interaction for cancer therapy appears to be quite promising, it is necessary to develop reagents that can induce anti-tumour activity without adverse effects such as elimination by phagocytosis of normal host cells highly expressing either CD47 or SIRPα. Moreover, there is the possibility that human SIPRα polymorphism, which affects binding of SIRPα to CD47, is a key determinant of efficient HSC engraftment or associated with pathogenesis of autoimmune diseases or any bone disorders. The investigation of whether SIRPα polymorphism is associated with the susceptibility of these diseases or affects efficiency of HSC transplantation might provide a new diagnostic test for human diseases and in determining eligibility for cell or organ transplantation.

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Conflict of Interest
None declared.

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Signal Regulatory Protein Alpha Is Present in Several Neutrophil Granule Populations and Is Rapidly Mobilized to the Cell Surface to Negatively Fine-Tune Neutrophil Accumulation in Inflammation

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Key Words
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Abstract
Signal regulatory protein alpha (SIRPα) is a cell surface glycoprotein with inhibitory functions, which may regulate neutrophil transmigration. SIRPα is mobilized to the neutrophil surface from specific granules, gelatinase granules, and secretory vesicles following inflammatory activation in vitro and in vivo. The lack of SIRPα signaling and the ability to upregulate SIRPα to the cell surface promote neutrophil accumulation during inflammation in vivo.

Introduction

Neutrophils constitute the frontline of defense in the innate immune system and migrate from blood into tissues in response to chemotactic and inflammatory factors [1]. This functional response relies on mobilization of membrane proteins to the plasma membrane, which takes place within minutes and is based on the exocytosis of cytoplasmic granules containing membrane proteins [2]. Neutrophils contain primary (azurophilic), secondary (specific), and tertiary (gelatinase) granules as well as a fourth subcellular compartment called secretory vesicles [2]. Granules and secretory vesicles are released following activation by chemotactic peptides, where the threshold for degranulation is such that secretory vesicles are released first, then tertiary granules, followed by secondary granules [2]. Primary granules are released by exocytosis only to a limited extent and rather fuse with phagosomes [2].

Signal regulatory protein alpha (SIRPα/SHPS-1/BIT/MFR/P84), a cell surface receptor of the immunoglobulin

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