CD47-Signal Regulatory Protein α (SIRPα) Regulates Fcγ and Complement Receptor–mediated Phagocytosis

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Abstract

In autoimmune hemolytic anemia (AIHA), circulating red blood cells (RBCs) opsonized with autoantibody are recognized by macrophage Fcγ and complement receptors. This triggers phagocytosis and elimination of RBCs from the circulation by splenic macrophages. We recently found that CD47 on unopsonized RBCs binds macrophage signal regulatory protein α (SIRPα), generating a negative signal that prevents phagocytosis of the unopsonized RBCs. We show here that clearance and phagocytosis of opsonized RBCs is also regulated by CD47-SIRPα. The inhibition generated by CD47-SIRPα interaction is strongly attenuated but not absent in mice with only residual activity of the phosphatase Src homology 2 domain–containing protein tyrosine phosphatase (SHP)-1, suggesting that most SIRPα signaling in this system is mediated by SHP-1 phosphatase activity. The macrophage phagocytic response is controlled by an integration of the inhibitory SIRPα signal with prophagocytic signals such as from Fcγ and complement receptor activation. Thus, augmentation of inhibitory CD47-SIRPα signaling may prevent or attenuate RBC clearance in AIHA.

Key words: macrophages • autoimmunity • anemia • red blood cells • SHP-1

Introduction

In autoimmune hemolytic anemia (AIHA),[1] "self" RBCs are opsonized with autoantibody and complement, and RBC phagocytosis is mediated primarily by Fcγ receptors, with some amplification from complement receptors (1–4). We recently found that unopsonized RBCs from CD47-deficient mice are recognized and rapidly phagocytosed by splenic red pulp macrophages in wild-type recipients. CD47 on normal RBCs binds to and activates signal regulatory protein α (SIRPα) on the macrophage, which inhibits macrophage activation and phagocytosis (5). Thus, red pulp macrophages have phagocytic receptors capable of recognizing normal "self" RBCs, and the CD47-SIRPα signal is sufficient to counteract this signal (5). It is unclear

how this capacity of CD47 (also known as integrin–associated protein [IAP]) is related to its function as a signaling molecule mediating the stimulatory effect of Arg-Gly-Asp (RGD)-containing matrix proteins on phagocytosis (6, 7).

Upon ligation and cross-linking, SIRPα is tyrosine phosphorylated by Src family kinases at its intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs [8]). These phosphorylated motifs bind the tyrosine phosphatases Src homology 2 domain–containing protein tyrosine phosphatase (SHP)-1 and SHP-2, thereby recruiting them to the cell surface and activating them. In macrophages, SHP-1 is the predominant ligand, but SHP-2 is also present (8). These phosphatases then interrupt signaling from tyrosine kinase–dependent receptors, e.g., the M-CSF receptor c-fms, likely by direct receptor dephosphorylation (9). Several other signaling molecules such as Grb2, son of sevenless (SOS), and the Src kinase Fgr also bind the phosphorylated SIRPα ITIM (9, 10). SIRPα when coligated with Fcε receptors prevents or attenuates Fcε receptor signaling (11). Also, the Src family kinase Fgr was found to be associated with SIRPα upon Fcγ receptor cross-linking in murine macrophages, re-

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[1]*Abbreviations used in this paper:* AIHA, autoimmune hemolytic anemia; BMM, bone marrow–derived macrophage; HSA, human serum albumin; IAP, integrin-associated protein; SHP, Src homology 2 domain–containing protein tyrosine phosphatase; SIRPα, signal regulatory protein α.

sulting in recruitment of SHP-1 to SIRPα and decrease in phagocytosis (10). However, the in vivo effect of SIRPα signaling in the context of Fc receptors is not known.

Although CD47^{−/−} RBCs are recognized and eliminated by splenic red pulp macrophages in vivo (5), the receptor mediating this positive recognition has not been identified. We will refer to this functional activity as the "RBC receptor." Due to the inhibitory signal generated by ligation of macrophage SIRPα by target CD47, wild-type RBCs are not eliminated (5). This leaves two important questions, relevant to AIHA, that were investigated in this study: first, does the CD47-SIRPα signal regulate only this "RBC receptor," or does it also affect the outcome of ligation of other phagocytic receptors? Second, is the outcome determined by the relative strength of the prophagocytic and inhibitory signals, or is the inhibitory signal dominant? We address these questions by determining the in vivo clearance and in vitro phagocytosis of wild-type and CD47^{−/−} RBCs opsonized with IgG or complement. The strength of the activating signal was regulated by controlling the level of opsonization, and SIRPα signaling was manipulated by regulating the amount of CD47 available on the target cell and by using mice and macrophages deficient in SHP-1 activity, a downstream mediator of SIRPα signaling in macrophages (8).

Materials and Methods

Reagents. Rat anti-murine CD47 (mIAP301; IgG2a; reference 7) was purified from tissue culture supernatant by ammonium sulfate precipitation and ion-exchange chromatography using DEAE sephacel (Amersham Pharmacia Biotech). Fab fragments of mIAP301 were prepared by papain digestion and purification using DEAE ion-exchange chromatography. Rat anti-murine SIRPα (P84; IgG1; reference 12) was purified from tissue culture supernatant by ammonium sulfate precipitation and protein-G chromatography using the mAb Trap II kit (Amersham Pharmacia Biotech). Human serum albumin (HSA) was from Alpha, Therapeutic Corp. Rabbit anti-murine RBC antiserum was from Cappel, and the rabbit anti-murine RBC IgG fraction was purified by protein-G chromatography (Amersham Pharmacia Biotech). Rat anti-murine CD14 (IgG1), rat anti-murine CD24 (IgM), and biotinylated anti-CD24 were from BD PharMingen. Avidin-FITC was from Molecular Probes. FITC-labeled goat anti–rabbit IgG and PKH26 were from Sigma-Aldrich. Chemicals were of the highest grade available and purchased from Sigma-Aldrich unless otherwise specified.

Mice. Male and female CD47^{−/−} C57BL/6J mice, backcrossed to C57BL/6J (The Jackson Laboratory) for 16 or more generations, and their heterozygous and homozygous littermates were from our own breeding colony (7). Motheaten viable mice (me^v/me^v) were obtained from The Jackson Laboratory, and Fcγ receptor^{−/−} mice were obtained from Taconics. CD18^{−/−} mice were a gift from Drs. D. Bullard (University of Alabama, Birmingham, AL) and A.L. Beaudet (Baylor College of Medicine, Houston, TX) (13). Animals were kept in accordance with National Institutes of Health and local guidelines and maintained in a specific pathogen-free barrier facility.

Opsonization of Murine RBCs. IgG-opsonized murine RBCs (RBC-IgG) were obtained by incubating washed RBCs with a 1:2 or 1:10 dilution (in vivo experiments; high and low opsonization, respectively) or a 1:5 dilution (in vitro experiments) of rabbit IgG anti-murine RBC (1 mg/ml) for 20 min at 37°C. For complement-opsonized murine RBCs (RBC-C3bi; reference 14), washed murine RBCs were first incubated with subagglutinating dilution of mAb rat IgM anti-CD24 (HSA) for 30 min at 37°C. Then, RBC-IgM were incubated in 5% C5-deficient mouse serum (The Jackson Laboratory) for 60 min at 37°C. For in vitro phagocytosis assays, opsonized RBCs were finally washed and resuspended in HBSS with 1% HSA at 5×10^8/ml. Opsonized RBCs for in vivo experiments were prepared as described below. Equivalence of opsonization levels on CD47^{+/+} and CD47^{−/−} RBCs was verified by flow cytometry, using FITC-labeled goat anti–rabbit IgG for IgG-opsonized RBCs or biotinylated anti-CD24 and avidin-FITC for complement-opsonized RBCs.

In Vivo Clearance of Unopsonized or Opsonized RBCs. Washed wild-type or CD47^{−/−} RBCs were stained with the fluorescent dye PKH26 (Sigma-Aldrich) according to the manufacturer's protocol and then washed three times in sterile PBS with 0.1% HSA. In experiments where IgG-opsonized RBCs (RBC-IgG) were used, PKH26-labeled RBCs were opsonized with rabbit IgG anti-murine RBCs as described above, and washed three times in sterile PBS. RBCs or RBC-IgG were finally resuspended in sterile pyrogen-free 0.9% NaCl to 30% (vol/vol). Recipient mice were given 200 μl of the stained RBCs or RBC-IgG and the clearance of fluorescent RBCs was followed using flow cytometry (Epics XL; Coulter Corp.) of 5-μl blood samples collected from a tail vein at the time points indicated. The fraction of fluorescent RBCs, of total number of recipient RBCs studied (100,000/sample), was determined. The data were normalized to the fraction at 30 min after injection (5). Use of the 5-min time point as a reference gave similar results, although the experimental variation was higher this early after injection (not shown).

Preparation of Bone Marrow Macrophages. Bone marrow was collected from femurs and tibias of donor mice, and bone marrow macrophages prepared as described previously (15).

RBC Phagocytosis in Bone Marrow Macrophages In Vitro. Phagocytosis was assessed using bone marrow macrophages in suspension (10). Washed macrophages (2×10^5) were centrifuged with 7.5 μl of RBC-IgG or 25 μl of RBC-C3bi for 1 min at 100 *g* and then gently resuspended in 100 μl of buffer. For phagocytosis of RBC-C3bi, 30 ng/ml of Phorbol dibutyrate (PDBu) and 1 mM MnCl₂ were added to stimulate ingestion (10). Where indicated, anti-CD47 (mAb mIAP301), anti-SIRPα (mAb P84), or control Ab (anti-CD14) were also added at 10 μg/2×10^5 macrophages. After incubation for 30 min at 37°C, uningested RBCs were removed by hypotonic lysis. Phagocytosis was assessed by phase contrast microscopy and quantitated as a phagocytic index (the number of RBCs ingested/100 macrophages).

Results

CD47 Increases Survival of IgG-opsonized RBCs In Vivo. Unopsonized CD47^{−/−} RBCs are recognized and eliminated by splenic red pulp macrophages in wild-type mice, which suggests that these macrophages have activating "RBC receptors" (5). This activating signal is under normal circumstances counteracted by an inhibitory signal resulting from CD47-SIRPα interaction (5). To determine if the CD47-SIRPα signal is also integrated with signals from
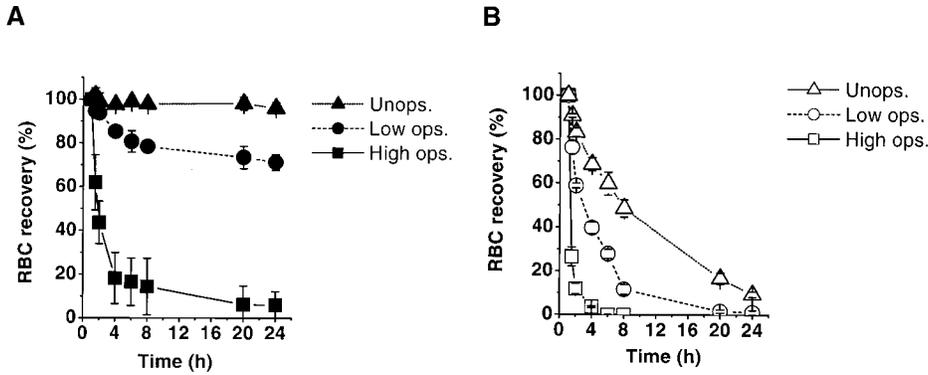
A

B

Unops.
Low ops.
High ops.

Figure 1. Clearance of IgG-opsonized RBCs in vivo is regulated by CD47 on the RBCs. Wild-type recipient mice were intravenously injected with PKH26-labeled wild-type RBCs (A; filled symbols) or CD47$^{-/-}$ RBCs (B; open symbols). RBCs were opsonized to different levels with rabbit polyclonal anti–mouse RBCs IgG to obtain low or highly opsonized (ops.) RBCs. Flow cytometric analysis of opsonized RBCs showed identical opsonization of wild-type and CD47$^{-/-}$ RBCs, and a fivefold difference in the level of opsonization between low- (circles) and high-opsonized (squares) RBCs. After extensive washing, 200 μl of low- or high-opsonized RBCs or unopsonized (Unops.) RBCs (triangles; 30% vol/vol in pyrogen-free 0.9% NaCl) was intravenously injected into wild-type recipient mice. Clearance of labeled RBCs was followed using flow cytometry of 5-μl blood samples collected from a tail vein at the time points indicated. Data are mean ± SD for three mice in each group. The rate of clearance increases with increased degree of opsonization and is at each level higher for CD47$^{-/-}$ targets than for CD47$^{+/+}$ RBCs.

other macrophage activating receptors, we investigated how CD47-SIRPα interaction affects macrophage Fcγ and complement receptor–mediated elimination of opsonized RBCs.

To investigate the interaction of CD47-SIRPα signaling with signals from macrophage Fcγ receptors in vivo, we opsonized CD47$^{-/-}$ and wild-type (CD47$^{+/+}$) RBCs to identical levels with rabbit anti–mouse RBC IgG. Opsonized RBCs were produced at both high and low levels of opsonization, with IgG on highly opsonized RBCs present at approximately five times the level detected on the low opsonized cells (by flow cytometry; not shown). At each level, opsonization of CD47$^{-/-}$ and wild-type RBCs was identical (assessed by flow cytometry; see Materials and Methods). Unopsonized CD47$^{-/-}$ RBCs are rapidly eliminated in wild-type recipients, whereas wild-type RBCs are not (5; Fig. 1). Increasing levels of opsonization accelerated the clearance of both CD47$^{-/-}$ and wild-type RBCs (Fig. 1). Thus, the rate of elimination was determined by both target cell CD47 and by the level of opsonization.

Virtually identical results were obtained in CD18$^{-/-}$ mice (13), suggesting that the increased rate of clearance obtained by IgG opsonization is mediated by macrophage Fcγ receptors, rather than by secondary effects from complement and complement receptors (not shown).

Phagocytosis of IgG-opsonized RBCs by Bone Marrow–derived Macrophages Is Regulated by CD47-SIRPα. Next, we turned to an in vitro system to more closely characterize the integration of Fcγ receptor and SIRPα signals in macrophages. Splenic red pulp macrophages phagocytose unopsonized RBCs, making it hard to isolate the effect of opsonization (5). Thus, we used bone marrow–derived macrophages (BMMs), which do not recognize unopsonized RBCs (5). Wild-type RBCs were opsonized with rabbit anti–mouse RBC IgG to a level resulting in moderate phagocytosis (93 ± 16 RBCs per 100 macrophages; Fig. 2 A). Identically opsonized CD47$^{-/-}$ RBCs yielded markedly higher levels of phagocytosis (398 ± 57 RBCs per 100 macrophages; Fig. 2 A). Anti-SIRPα mAb P84 increased phagocytosis of wild-type, but not of CD47$^{-/-}$
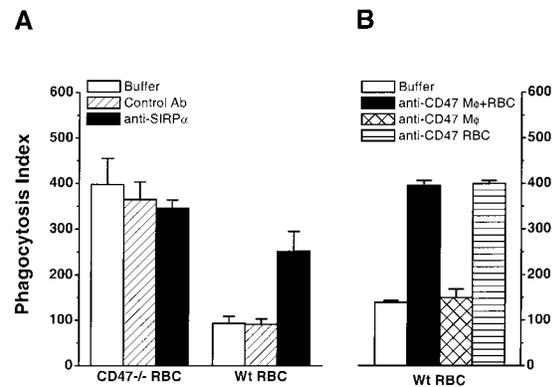
A

B

Figure 2. Target cell CD47 inhibits Fcγ receptor–mediated phagocytosis of opsonized RBCs in BMMs via ligation of macrophage SIRPα. (A) Effects of anti-SIRPα mAb P84 on phagocytosis of IgG-opsonized RBCs in CD47 wild-type (Wt) BMMs was assayed in medium alone (white bars), in the presence of 10 μg anti-SIRPα mAb P84 (black bars), or in the presence of control mAb anti-CD14 (hatched bars). Wild-type RBCs are phagocytosed at a lower rate than CD47$^{-/-}$ RBCs, which can be virtually completely corrected by anti-SIRPα mAb P84. The rate of CD47$^{-/-}$ RBC phagocytosis can be significantly enhanced by increased opsonization (not shown). Thus, the lack of anti-SIRPα effect on CD47$^{-/-}$ RBC phagocytosis is not due to saturation of the system. (B) Effects of anti-CD47 mAb mIAP301 on phagocytosis of IgG-opsonized wild-type RBCs in wild-type BMMs. Macrophages (Mφ) and RBCs were incubated in medium alone (white bar) or in the presence of 10 μg Fab fragments of anti-CD47 mAb mIAP301 (black bar). To further separate the anti-CD47 blocking effect on macrophage CD47 from that on RBC CD47, respectively, macrophages only (cross-hatched bar) or RBCs only (hatched bar) were preincubated with 10-μg Fab fragments of anti-CD47 mAb mIAP301, extensively washed, and then mixed for assay of phagocytosis. CD47$^{-/-}$ or wild-type RBCs were opsonized to identical levels with rabbit anti–mouse RBC IgG and then added to macrophages (2 × 10^5) in suspension (100 μl). After 30 min at 37°C, uningested RBCs were lysed and phagocytosis was determined by light microscopy. Results are expressed as number of RBCs ingested per 100 macrophages (phagocytosis index) and are mean ± SEM for three separate experiments. Ligation of target cell CD47 enhances the phagocytosis of wild-type RBCs to the level seen with CD47$^{-/-}$ RBCs.

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RBCs, whereas an isotype-matched control mAb (anti-CD14) was without effect (Fig. 2 A). The level of CD47$^{-/-}$ RBC phagocytosis could be increased significantly by increased opsonization (beyond 800 RBCs per 100 macrophages), showing that the lack of anti-SIRPα effect was not due to an overall saturation of the system. The level of phagocytosis of wild-type RBCs in the presence of anti-SIRPα was not significantly different from that obtained with CD47$^{-/-}$ RBCs. Thus, phagocytosis of wild-type and CD47$^{-/-}$ RBCs becomes equivalent when CD47-SIRPα interaction is prevented by blocking macrophage SIRPα. This implies that blockade of target cell CD47 would also enhance phagocytosis of wild-type RBCs to the level seen with CD47$^{-/-}$ targets.

To test this hypothesis, we added saturating amounts of anti-CD47 mAb mIAP301 Fab fragments, which block CD47-SIRPα interaction in vitro (not shown). As expected, phagocytosis of wild-type RBCs was enhanced (Fig. 2 B). mAb mIAP301 Fab acted by blocking of CD47 on the target, whereas blocking of macrophage CD47 was without effect (Fig. 2 B). Thus, SIRPα signals resulting from interaction with target cell CD47 can attenuate the phagocytic signal from macrophage Fcγ receptors.

Phagocytosis of Complement-opsonized RBCs Is Also Controlled by CD47-SIRPα. Complement receptors are a second class of macrophage phagocytic receptors (16). To study the role of CD47 in complement receptor–mediated phagocytosis, we opsonized CD47$^{-/-}$ and wild-type RBCs with C3bi (see Materials and Methods), then incubated them with wild-type BMMs. As shown in Fig. 3,
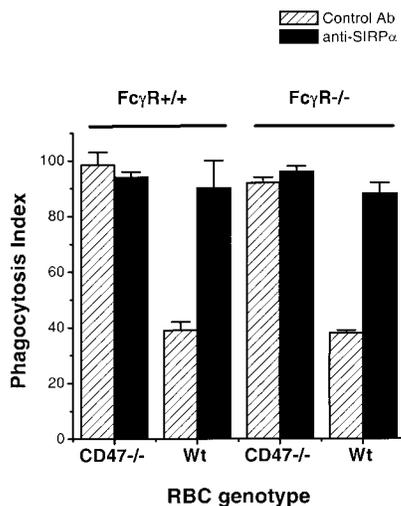
C3bi-opsonized CD47$^{-/-}$ RBCs were phagocytosed at a significantly higher rate than wild-type RBCs. Phagocytosis of wild-type RBCs could be augmented to the level of CD47$^{-/-}$ RBC phagocytosis by the addition of SIRPα-blocking mAb P84. The level of phagocytosis was the same in Fcγ receptor–deficient macrophages (Fig. 3), showing that this system is independent of Fcγ receptors. Thus, the phagocytic signals from Fcγ receptors, complement receptors, and the RBC receptor are integrated with the inhibitory signal from SIRPα. None of the signals is dominant. Rather, the outcome is determined by the relative signal strengths from prophagocytic and inhibitory receptors.

The Tyrosine Phosphatase SHP-1 Is the Main Mediator of SIRPα Signaling. SHP-1 phosphatase activity is ~80% reduced in motheaten viable (me^v/me^v) compared with wild-type mice (17–19). SIPRα has been shown to be the major substrate of SHP-1 (8), and SHP-1 is the major phosphatase associated with SIRPα in macrophages, although SHP-2 predominates in other cells (9, 20, 21). If CD47-SIRPα signaling in macrophages is mediated by SHP-1, me^v/me^v mice should lack or show strong reduction of the inhibitory signal mediated by CD47 on target RBCs. Thus, me^v/me^v mice should eliminate opsonized wild-type RBCs at accelerated rates similar to those seen with CD47$^{-/-}$ targets.

To test the role of SHP-1 in CD47-SIRPα signaling, we examined the clearance of low-opsonized (see for example Fig. 1) wild-type and CD47$^{-/-}$ RBCs in me^v/me^v mice. Consistent with our hypothesis, clearance of opsonized wild-type RBCs in these recipients was independent of CD47 and accelerated to the rate seen with CD47$^{-/-}$ targets (Fig. 4). Again, we wished to verify our results in vitro. As shown in Fig. 5, IgG-opsonized RBCs were phagocytosed by me^v/me^v BMMs without regard to the presence or absence of CD47 on the target cells, and at the same rate as wild-type BMM phagocytosed CD47$^{-/-}$ RBCs. Fur-
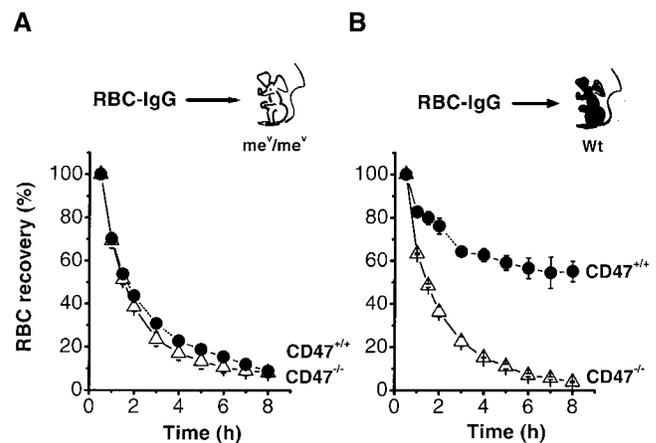

Figure 3. Phagocytosis of C3bi-opsonized RBCs is regulated by target cell CD47 via macrophage SIRPα. BMM (2 × 10^5) and C3bi-opsonized wild-type (Wt) or CD47$^{-/-}$ RBCs, in the presence of anti-SIRPα mAb P84 (10 μg) or control anti-CD14 mAb (10 μg), were incubated with 30 ng/ml of PDBu and 1 mM MnCl$_2$ in a 100-μl volume for 30 min at 37°C. Uningested RBCs were lysed and phagocytosis was determined by light microscopy. Phagocytosis index is number of RBCs ingested per 100 macrophages and is expressed as mean ± SEM for three separate experiments. To exclude any role of Fcγ receptors, the same experiments were also performed in Fcγ receptor–deficient BMMs.

Figure 4. Fcγ receptor–mediated clearance of IgG-opsonized RBCs is independent of target cell CD47 in SHP-1–deficient motheaten viable (me^v/me^v) mice. (A) me^v/me^v or (B) wild-type (Wt) mice were intravenously injected with PKH26-labeled IgG-opsonized wild-type (●) or CD47$^{-/-}$ (△) RBCs. Opsonization of RBCs and clearance of injected RBCs was performed and followed as described in the legend to Fig. 1. Data are mean ± SD for three mice in each group.

thermore, anti-SIRPα mAb P84 had no effect on phagocytosis by me^v/me^v macrophages. These results are consistent with the in vivo results shown in Fig. 4. Together, the data presented suggest that the SIRPα signal in me^v/me^v macrophages is absent or highly attenuated.

If SIRPα signals exclusively via the SHP-1 phosphatase, SIRPα is the only receptor for CD47, and CD47 is the only difference between wild-type (CD47$^{+/+}$) and CD47$^{-/-}$ RBCs, me^v/me^v mice should show reduced ability to distinguish between wild-type and CD47$^{-/-}$ RBCs also in the absence of opsonization. Surprisingly, the clearance of the two RBC types is as different in me^v/me^v as in wild-type mice (Fig. 6). Likely, the residual phosphatase activity of SHP-1 in me^v/me^v mouse macrophages is sufficient to allow some signaling from CD47-SIRPα interaction. This signal would be strong enough to counteract the phagocytic signal from the RBC receptor. Alternatively, CD47-SIRPα signaling may to a small extent be mediated independent of SHP-1 phosphatase activity, e.g., via SHP-2. Additional CD47-SIRPα-independent pathways are very unlikely, as both anti-SIPRα and anti-CD47 can block the entire difference between the two RBC types (5; Figs. 2 and 3).

Heterozygote (CD47$^{+/-}$) and wild-type (CD47$^{+/+}$) RBCs behaved identically in all experiments performed in wild-type and heterozygote recipients (5; Fig. 6). This suggests that the signal from CD47-SIRPα is significantly stronger than that from the RBC receptor so that even half the normal CD47 density suffices to completely suppress clearance. Interestingly, CD47$^{+/-}$ RBCs in me^v/me^v mice are cleared rapidly, albeit slower than CD47$^{-/-}$ RBCs (Fig. 6). This further suggests that the inhibitory signal obtained from CD47-SIRPα interaction in me^v/me^v mice is much
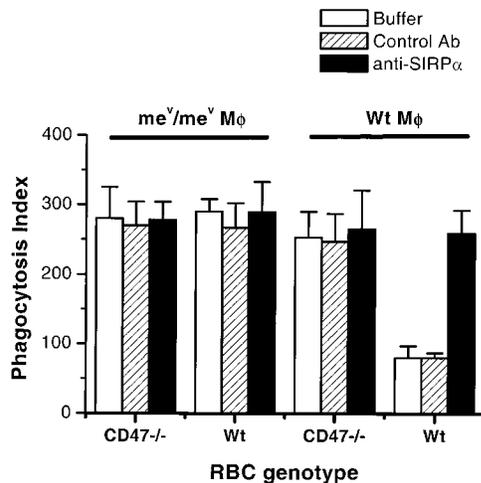
Figure 6. Residual and attenuated inhibitory CD47-SIRPα signal in me^v/me^v mice. Clearance of unopsonized PKH26-labeled CD47$^{+/+}$ (●), CD47$^{+/-}$ (■), or CD47$^{-/-}$ (△) RBCs in (A) me^v/me^v or (B) wild-type (Wt) mice. Clearance of unopsonized RBCs was performed as described in the legend to Fig. 1. Data are mean ± SD for three to five mice in each group.

weaker than it is in wild-type mice. When RBCs are sufficiently opsonized to also obtain some phagocytosis of wild-type RBCs, the SIRPα signal in the me^v/me^v background must be relatively insignificant compared with the Fcγ receptor signal, as no difference was observed between IgG-opsonized wild-type and CD47$^{-/-}$ target cells in me^v/me^v mice (Fig. 4).

Discussion

Activation of phagocytosis in macrophages is mediated by several different receptors, including IgG Fcγ receptors and complement receptors (22). Fcγ and complement receptors also play a major role in cell clearance in autoimmune processes, such as AIHA. We have recently shown that CD47, a ubiquitously expressed membrane glycopro-

Figure 5. Phagocytosis of IgG-opsonized RBCs is independent of CD47 in BMMs from SHP-1–deficient me^v/me^v mice. BMMs (Mϕ) from me^v/me^v or wild-type (Wt) mice were incubated with IgG-opsonized RBCs in medium alone (white bars), in the presence of 10 μg anti-SIRPα mAb P84 (black bars) or control anti-CD14 mAb (hatched bars). Phagocytosis was determined as described in the legend to Fig. 2. Data are presented as mean ± SEM for three separate experiments.
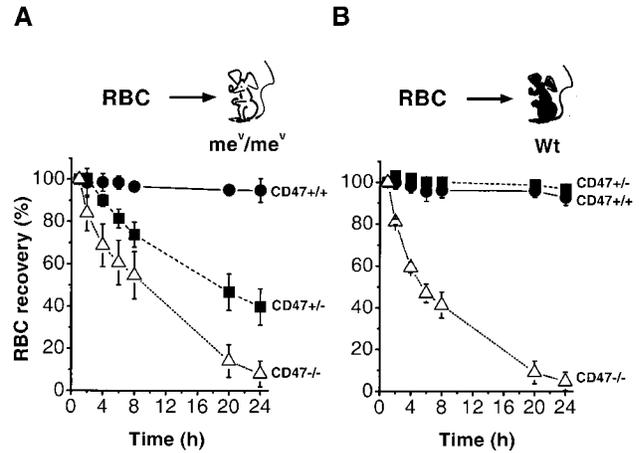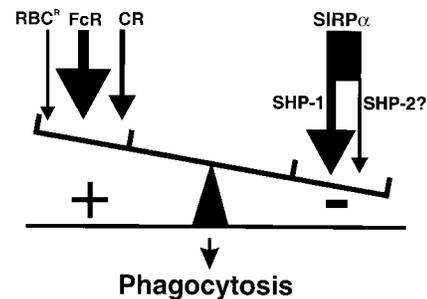
Figure 7. A model for the regulation of macrophage phagocytosis based on a summation of positive prophagocytic and negative CD47-SIRPα–generated signals. Neither type of signal is dominant. Rather, the decision to phagocytose is based on the summation of weak positive signals (the RBC receptor), strong positive signals (Fc and complement receptors), and negative signals (SIRPα). The majority of SIRPα signaling is mediated via SHP-1 as shown by the marked reduction of the signal in me^v/me^v mice. The data presented do not rule out the possibility that a small subset of the SIRPα signal may be mediated by SHP-2.

tein, functions as a marker of "self" on RBCs, and that unopsonized murine CD47$^{-/-}$ RBCs are rapidly recognized and cleared by splenic red pulp macrophages in wild-type recipients (5). Under normal circumstances, CD47 on RBCs prevents their elimination by ligating the inhibitory macrophage receptor SIRPα (5). The data also suggested that splenic, but not BMMs, have "RBC receptors" capable of recognizing circulating RBCs (5).

Both CD47$^{-/-}$ and wild-type RBCs are more rapidly cleared in vivo and more rapidly phagocytosed in vitro as the level of IgG opsonization is increased (Figs. 1 and 2). At all levels, CD47$^{-/-}$ RBC are cleared more rapidly than wild-type cells. This demonstrates that the inhibitory CD47-SIRPα signal is integrated with the Fcγ receptor signal proximal to the decision to phagocytose, and that neither the SIRPα nor the Fcγ receptor signal is dominant. Rather the outcome is determined by the relative strength of the two signals. RBCs opsonized with the complement fragment C3bi are bound and phagocytosed via the CR3/$\alpha_M\beta_2$/Mac-1 integrin (16). We also show that complement-mediated phagocytosis is regulated by the CD47-SIRPα signal and that again neither signal is dominant (Fig. 3). It is well known that CR3 and Fcγ receptors act synergistically (23–26). In vivo, RBCs that are opsonized with very low levels of IgG are not cleared in the absence of complement (27). Low levels of complement deposition alone also fail to trigger phagocytosis, whereas combination of the two results in rapid clearance in vivo and phagocytosis in vitro (25–27). Thus, phagocytosis is based on the summation of positive signals from at least Fcγ receptors, complement receptors, and the so far uncharacterized "RBC receptors," with the negative signal from SIRPα.

SHP-1 phosphatase activity in me^v/me^v mice is only ~20% of normal, whereas SHP-2 activity is unaffected by the mutation (18, 19). The altered RBC clearance in me^v/me^v mice suggested strongly attenuated SIRPα signaling arguing that the majority of the CD47-SIRPα signal is dependent on SHP-1 phosphatase activity. This is consistent with biochemical data for SIRPα-SHP-1 colocalization, the relative abundance of SHP-1 over SHP-2 in macrophages, and signaling data both in macrophages and in model systems (8, 11, 28). Although the data suggest that SHP-1 is responsible for the majority of CD47-SIRPα signaling, they do not rule out an accessory role for SHP-2. The signal resulting from CD47-SIRPα interaction in me^v/me^v mice is insignificant compared with the activating signal from the Fcγ receptor even with lightly opsonized RBCs (Fig. 4). In contrast, even this attenuated signal suffices to counteract the "RBC receptor" on red pulp macrophages, as in me^v/me^v mice wild-type levels of CD47 protect from clearance (Fig. 6). The protection is tenuous, as demonstrated by the fact that a reduction of CD47 on RBCs by as little as 50% leads to their rapid elimination in me^v/me^v mice. In wild-type mice with normal SIRPα signaling, much higher reduction in CD47 levels are required before target RBCs are significantly cleared (Fig. 6, and unpublished data).

Fig. 7 is a schematic depiction of the semiquantitative implications of our data. The SIRPα signal generated by

CD47 on normal RBCs is significant and normally counteracts the weak prophagocytic RBC receptor signal. A significant signal from Fcγ and/or complement receptors is required to overcome the normal SIRPα signal. This inhibitory signal is absent when the target particles are CD47 deficient or attenuated when downstream signaling is reduced such as in me^v/me^v mice.

Self versus nonself discrimination is a cornerstone in host defense. CD47-SIRPα interaction provides such discrimination by enhancing the contrast between self and nonself. A self (CD47$^+$) particle can escape phagocytosis even when it is opsonized to a degree that for a nonself (CD47$^-$) particle would lead to rapid phagocytosis. In type II inflammatory diseases involving cytotoxic self-reactive Abs, Fcγ receptor–mediated mechanisms are known to be of fundamental importance (1). Our data shows that CD47-SIRPα interacts with at least Fcγ and complement receptor signals and that modulation of CD47-SIRPα signaling has drastic effects on the outcome at all but extreme levels of opsonization. This should make CD47-SIRPα appealing as a means to modulate autoimmune destruction in AIHA and immune thrombocytopenia (ITP), where sensitized blood cells are cleared by splenic macrophages.

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