Lack of CD47 on nonhematopoietic cells induces split macrophage tolerance to CD47^{null} cells

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Macrophages recognize CD47 as a marker of "self" and phagocytose CD47^{null} hematopoietic cells. Using CD47 chimera models, here, we show that the phagocytic activity of macrophages against CD47^{null} hematopoietic cells is conferred by CD47 expression on nonhematopoietic cells, and this "education" process is hematopoietic cell-independent. Macrophages in the chimeras where nonhematopoietic cells express CD47 phagocytose CD47^{null} cells, whereas those in the chimeras lacking CD47 on nonhematopoietic cells are tolerant to CD47^{null} cells. However, macrophages in the latter chimeras retain phagocytic activity against CD47^{null} RBCs, demonstrating a split macrophage tolerance to CD47^{null} hematopoietic cells. The findings highlight the potential importance of nonhematopoietic cells in the regulation of macrophage function, and suggest a previously uncharacterized mechanism of macrophage tolerance.

chimera | phagocytosis | signaling regulatory protein-α

Macrophages are essential to both innate and adaptive immune responses. They initiate the innate immune response by recognizing pathogens by means of a range of receptors with specificity for pattern-recognition molecules (1, 2). Macrophages also express inhibitory receptors that control macrophage activity within tissues (3). One of these receptors called signaling regulatory protein (SIRP)α (also known as CD172a, SHPS-1) is expressed on macrophages and dendritic cells (DCs), which recognizes CD47, a pentaspan membrane glycoprotein ubiquitously expressed in all tissues (4–6). Previous studies have shown that CD47 is an essential marker of "self," and its signaling through SIRPα prevents inappropriate self-phagocytosis (7). Hematopoietic cells not expressing CD47 are rapidly cleared from the bloodstream by macrophages and DCs (7, 8) [supporting information (SI) Fig. 6]. Because only CD47 KO cells are cleared in WT mice receiving a mixture of CD47 KO and WT cells, CD47 expression on an individual cell, but not on macrophages or other surrounding cells, is required for inhibition of phagocytosis, and such a protection is likely mediated by a direct interaction between CD47 on a target cell and SIRPα on macrophages (SI Fig. 7).

Interestingly, macrophages and DCs from CD47 KO mice do not phagocytose CD47 KO cells. Because the level of SIRPα expression on CD47 KO macrophages is virtually the same as that of WT macrophages, this tolerance to CD47 KO cells is unlikely induced at the level of SIRPα expression (7). It is possible that lack of CD47 expression on macrophages or in the environment may regulate macrophage function. Alternatively, such tolerance might be acquired during the early stage of prenatal development as compensation for the disrupted gene, and may not occur in normal macrophages. Furthermore, it remains unknown why CD47, but not the ligands for other inhibitory receptors, is used by macrophages to identify self in WT mice. To address these questions and to understand the mechanisms of macrophage tolerance, here, we have evaluated the phagocytic activity against CD47 KO cells of macrophages from various types of CD47 chimeras, in which CD47 is expressed on hematopoietic cells, nonhematopoietic cells, or on cells of both compartments.

Results

Tolerization of WT Mouse Macrophages to CD47 KO Cells in CD47 Chimeras Prepared by Injecting WT Mouse Bone Marrow Cells into CD47 KO Mice. To determine whether macrophages developing from normal WT (i.e., CD47$^{+/+}$) stem cells in the presence of CD47 KO cells can become tolerant to CD47 KO cells *in vivo* and to understand the role of hematopoietic vs. nonhematopoietic cells in regulation of macrophage function, we followed phagocytic activity of macrophages developing in various types of CD47 chimeras. We first generated mixed CD47 chimeras, in which hematopoietic cells are composed of both WT and CD47 KO cells, but all nonhematopoietic cells lack CD47 expression, by transferring lineage-negative (Lin$^-$) bone marrow cells (BMCs) of WT mice into sublethally irradiated syngeneic CD47 KO mice. FACS analysis of peripheral blood mononuclear cells (PBMCs) showed that all recipient mice maintained stable mixed hematopoietic chimerism for >40 weeks after bone marrow transplantation (BMT), demonstrating successful engraftment and differentiation of WT donor marrow stem cells in CD47 KO mice (Fig. 1 *A–C*). Mixed chimerism was also detected in other tissues (e.g., spleen and lymph nodes) of these chimeras (Fig. 1*D*). The long-term coexistence of WT and CD47 KO cells in these mice suggests that WT macrophages and DCs developing in the presence of CD47 KO cells were tolerized to CD47 KO cells. To confirm this possibility, we performed clearance assays to assess the ability of macrophages and DCs to clear CD47 KO cells. CFSE-labeled CD47 KO splenocytes were rapidly cleared in WT controls, but not in mixed chimeras (Fig. 1 *E* and *F*). The levels of CFSE-labeled CD47 KO cells in mixed chimeras were comparable to those in CD47 KO controls. Furthermore, in the mixed chimeras that received a 1:1 mixture of CFSE-labeled WT and PKH26-labeled CD47 KO splenocytes, the levels of injected WT and CD47 KO cells were comparable over time (Fig. 1 *G* and *H*), similar to that observed in CD47 KO mice (Fig. 1*H* and SI Fig. 7). In contrast, PKH26-labeled CD47 KO cells were rapidly eliminated in the WT control mice (Fig. 1*H* and SI Fig. 7).

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Abbreviations: BMC, bone marrow cell; BMT, bone marrow transplantation; DC, dendritic cell; NK, natural killer; PBMC, peripheral blood mononuclear cell; SIRP, signaling regulatory protein.

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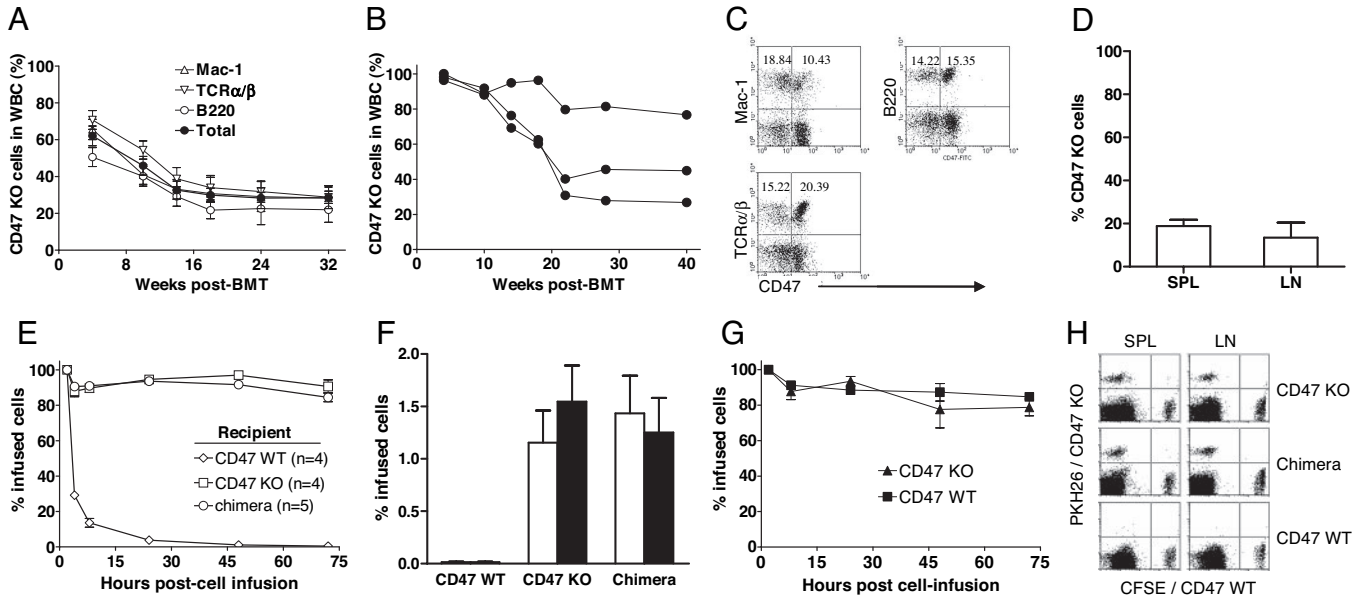

Fig. 1. Macrophage tolerance to cells missing CD47 in mixed hematopoietic chimeras created by transferring WT Lin$^-$ BMCs into sublethally (6 or 3 Gy)-irradiated CD47 KO mice. (*A–D*) Long-term mixed hematopoietic chimerism in CD47 KO recipients of WT Lin$^-$ BMCs. (*A*) Multilineage mixed hematopoietic chimerism in 6 Gy-irradiated CD47 KO recipients of WT Lin$^-$ BMCs. Shown are percentages (mean ± SDs; $n = 7$) of TCR$\alpha\beta^+$ (T cells), B220$^+$ (B cells), Mac-1$^+$ and total CD47 KO WBCs at the indicated times. (*B*) Levels of CD47 KO WBCs in 3-Gy-irradiated CD47 KO recipients of WT Lin$^-$ BMCs. Each line represents an individual animal. (*C*) Flow cytometry profiles showing mixed hematopoietic chimerism in WBCs of a representative chimera. (*D*) Six-Gy-irradiated mixed chimeras (the same mice shown in Fig. 1 *A*; $n = 7$) were killed at week 32 after BMT, and percentages of CD47 KO cells in spleen (SPL) and lymph nodes (LN) were determined by flow cytometry. (*E–H*) Lack of clearance of CD47 splenocytes in the chimeras. (*E* and *F*) At week 24 after BMT, chimeras were injected with 3×10^7 CFSE-labeled CD47 KO B6 splenocytes, and the clearance of injected CFSE-labeled CD47 KO cells was determined by flow cytometry. Age-matched WT and CD47 KO B6 mice that did not receive BMT were used as controls. (*E*) Levels (mean ± SDs) of injected CFSE$^+$ CD47 KO cells in peripheral blood at the indicated times. (*F*) Levels (mean ± SDs) of injected CFSE$^+$ CD47 KO cells in SPL (□) and LN (■) at day 4 after cell transfer. (*G* and *H*) A mixture (1:1) of CFSE-labeled WT and PKH26-labeled CD47 KO splenocytes (2.5×10^7 each) were injected into mixed chimeras ($n = 3$) at week 24 after BMT, and the clearance of injected cells was determined by flow cytometry. (*G*) Levels (mean ± SDs) of CFSE$^+$ WT (■) and PKH26$^+$ CD47 KO (▲) cells in peripheral blood of chimeras at the indicated times. (*H*) FACS profiles showing the levels of CFSE$^+$ WT and PKH26$^+$ CD47 KO cells in SPL and LN of a representative chimera and age-matched WT and CD47 KO B6 control mice at day 4 after cell transfer. Results in Fig. 1 *E* and *G* are normalized with the values at 2 h after cell transfer as 100%.

Lack of CD47 on Nonhematopoietic Cells Alone Is Sufficient to Induce Macrophage Tolerance to CD47 KO Cells. To further understand the role of hematopoietic vs. nonhematopoietic cells in the development of macrophage tolerance, we created full WT hematopoietic chimeras where all hematopoietic cells express CD47, by injecting WT Lin$^-$ BMCs into lethally irradiated CD47 KO mice. Lethally irradiated WT mice receiving Lin$^-$ WT BMCs (WT → WT chimeras) were used as controls. All lethally irradiated CD47 KO recipients of WT Lin$^-$ BMCs (WT → KO chimeras) lost CD47 KO hematopoietic cells by 4 weeks, with the exception that low levels of CD47 KO TCR$\alpha\beta$ T cells remained detectable for ≈12 weeks (Fig. 2*A*). At week 24 after transplantation when full WT donor hematopoietic chimerism was established in WT → KO chimeras, we injected CFSE-labeled CD47 KO splenocytes into these mice and WT → WT chimera controls. Macrophages in WT → WT chimeras, but not those in full WT → KO chimeras were phagocytic against CD47 KO cells (Fig. 2*B*).

To further assess the phagocytic activity of macrophages in these chimeras, we injected CD47 KO BMCs into these full chimeric mice after conditioning with sublethal (3 or 6 Gy) irradiation. CD47 KO BMCs were able to engraft and produce multilineage hematopoietic cells in WT → KO, but not in WT → WT chimeras. CD47 KO cells (including T, B and Mac-1$^+$ cells; data not shown) were detected in various tissues of all WT → KO chimeras, but were absent in WT → WT chimeras (Fig. 2 *C* and *D*). Taken together, these data demonstrate that nonhematopoietic cells can regulate macrophage function and induce macrophage tolerance independently of hematopoietic cells.

Lack of CD47 Expression on Hematopoietic Cells Alone Failed to Induce Macrophage Tolerance to CD47 KO Cells. To test the potential of hematopoietic cells to induce macrophage tolerance to CD47 KO cells, we next created mixed CD47 chimeras composing of WT and CD47 KO hematopoietic cells but only CD47$^{+/+}$ nonhematopoietic cells, by injecting CD47 KO BMCs into sublethally irradiated WT mice. To prevent initial rejection of CD47 KO cells by recipient macrophages, all recipient mice were treated with Cl_2MDP-liposomes (9) to deplete macrophages (0.02 mg/g of body weight at day −2, and 0.01 mg/g body weight at days 3, 6, and 11 with respect to BMT). Although initial engraftment of CD47 KO cells was achieved in these mice, the levels of CD47 KO donor chimerism declined rapidly between 4 and 8 weeks after transplantation and eventually disappeared (Fig. 3*A*). Of note, CD47 KO donor T cells were not detected at any time. Because the thymus contains abundant phagocytes (for clearing large numbers of nonselected and negatively selected thymocytes) (10), and macrophage recovery was likely to take place in the thymus before *de novo* T cell development from CD47 KO donor marrow cells in these chimeras, the lack of CD47 KO T cells in these mice is presumably because of the clearance of CD47 KO thymocytes or their progenitors in the thymus. Because macrophages in bone marrow are less effective in phagocytosis (7, 11), the relatively long-term presence of bone marrow-derived (especially Mac-1$^+$) CD47 KO cells in the blood of these chimeras could be due to the slow clearance of CD47 KO BMCs in these mice. In support of this possibility, we observed that the level of CD47 KO cells in bone marrow was markedly greater than in blood, spleen and thymus in these chimeras (SI Fig. 8).

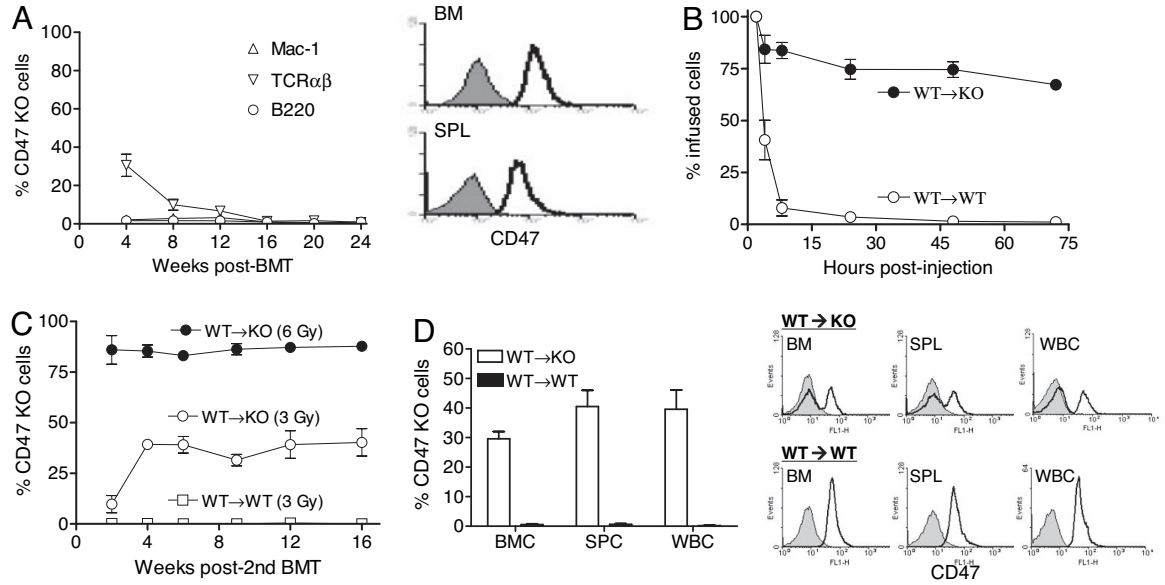

Fig. 2. Lack of CD47 expression on nonhematopoietic cells induces macrophage tolerance to CD47 KO cells. Full donor hematopoietic chimeras were created by injection of WT Lin$^-$ BMCs into lethally irradiated CD47 KO (WT → KO) or WT (WT → WT) mice. (*A*) Flow cytometry analysis of hematopoietic chimerism in CD47 KO recipients of WT Lin$^-$ BMCs (i.e., WT → KO chimeras). (*Left*) Levels (mean ± SDs; $n = 9$) of CD47 KO Mac-1$^+$, TCR$\alpha\beta^+$, and B220$^+$ cells in WBCs at the indicated times after BMT. (*Right*) Representative flow cytometry profiles showing lack of CD47 KO cells in bone marrow (BM) and spleen (SPL) at week 24 after BMT (all these WT → KO chimeras showed full donor hematopoietic chimerism). Open and shaded histograms represent staining with anti-CD47 and isotype control mAb, respectively. (*B*) Clearance of CFSE-labeled CD47 KO splenocytes in WT → KO chimeras with full WT donor hematopoietic chimerism (●; $n = 3$) and WT → WT chimeras (○; $n = 3$) at week 24 after BMT (the clearance assay was performed as described in Fig. 1*E*). (*C* and *D*) Full WT → KO and WT → WT chimeras received sublethal irradiation (3 or 6 Gy), followed by second BMT (5×10^5 Lin$^-$ BMCs) from CD47 KO mice ($n = 3$ per group). (*C*) Percentages (mean ± SDs) of CD47 KO cells in WBCs of WT → KO and WT → WT chimeras at the indicated times after second CD47 KO BMT. (*D*) Levels (mean ± SDs) of CD47 KO cells in BM, spleen, and WBCs of 3-Gy-irradiated WT → KO and WT → WT chimeras at week 32 after second CD47 KO BMT. Representative flow cytometry profiles (*Upper*, WT → KO; *Lower*, WT → WT) are shown on right (open and shaded histograms are staining with anti-CD47 and isotype control mAb, respectively).

To confirm whether the decline of CD47 KO cells in these mice was due to phagocytosis, but not to hematopoietic defects of CD47 KO BMCs, we injected CFSE-labeled CD47 KO mouse splenocytes into these chimeras at weeks 24 and 54 after BMT, and followed the clearance of injected CD47 KO cells by flow cytometry. Clearance of injected CFSE$^+$ CD47 KO cells was clearly seen in the chimeric mice, but not in CD47 KO control mice (Fig. 3*B*). The delayed clearance of injected CD47 KO cells in the chimeras at week 24 compared with WT mouse controls (Fig. 3*B Left*) was presumably due to the dilution of injected CD47 KO cells by the endogenous CD47 KO cells in the chimeras, i.e., both injected CFSE$^+$ and endogenous CD47 KO cells were being phagocytosed by macrophages in the chimeras, whereas injected CFSE$^+$ CD47 KO cells were the only CD47 KO cells being phagocytosed by macrophages in WT mouse controls. Consistent with this possibility, WT mouse controls and chimeras showed comparable clearance of injected CFSE$^+$ CD47 KO cells at week 54 (Fig. 3*B Right*), when the chimeras lost CD47 KO donor chimerism. These results indicate that macrophages developing *de novo* in the presence of CD47 KO hematopoietic cells retained the ability to phagocytose CD47 KO cells. Therefore, it is the absence of CD47 expression on nonhematopoietic cells that is required for the induction of macrophage tolerance to CD47 KO cells.

Split Macrophage Tolerance to CD47 KO Cells in CD47 Chimeras. Surprisingly, the lack of CD47 expression on nonhematopoietic cells failed to prevent phagocytosis of CD47 KO RBCs. In mixed WT → CD47 KO chimeras (Fig. 1*A*), where CD47 KO cells, including T cells, B cells, and Mac-1$^+$ cells, were durably maintained, CD47 KO RBCs declined rapidly and became undetectable by 12 weeks after injection of WT Lin$^-$ BMCs (Fig. 4*A*). Furthermore, injection of CD47 KO BMCs into full WT →

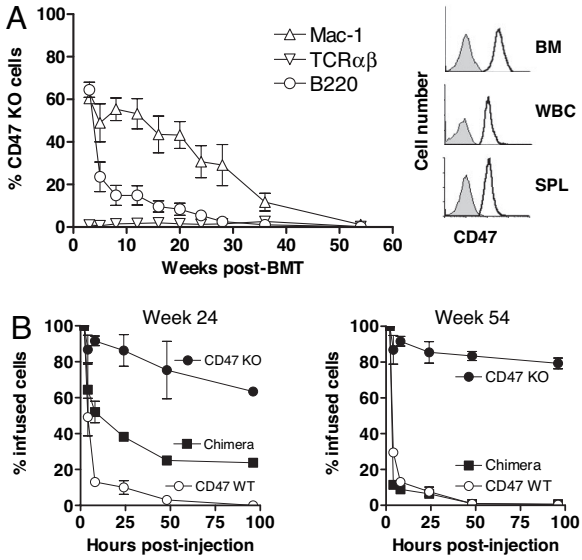

Fig. 3. Elimination of CD47 KO cells by macrophages in CD47 KO → WT bone marrow chimeras where nonhematopoietic cells express CD47. (*A*) Loss of CD47 KO cells in 6-Gy-irradiated WT recipients of CD47 KO Lin$^-$ BMCs ($n = 5$). (*Left*) Percentages (mean ± SDs) of CD47 KO Mac-1$^+$, TCR$\alpha\beta^+$, and B220$^+$ cells in WBCs at the indicated times. (*Right*) Representative flow cytometry profiles showing the absence of CD47 KO cells in the indicated tissues at week 54 (CD47 KO cells were undetectable in all of the recipients). Open and shaded histograms represent staining with anti-CD47 and isotype control mAb, respectively. (*B*) Clearance of CFSE-labeled CD47 KO splenocytes in these CD47 KO → WT bone marrow chimeras (■; $n = 5$) and age-matched WT (○; $n = 3$) and CD47 KO (●; $n = 3$) controls at week 24 (*Left*) and week 54 (*Right*) after BMT (the clearance assay was performed as described in Fig. 1*E*).

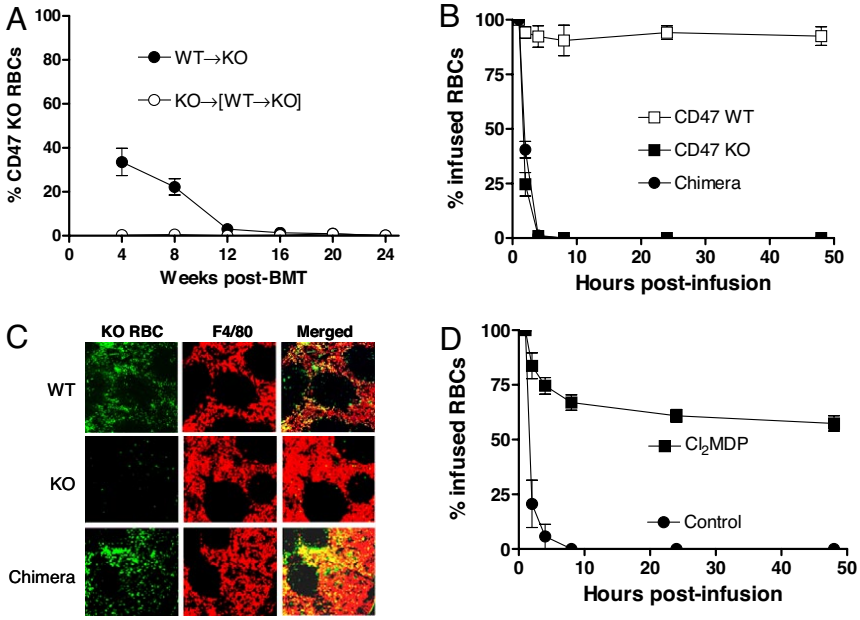

Fig. 4. Clearance of CD47 KO RBCs in CD47 chimeras. (*A*) Loss of CD47 KO RBCs in WT → CD47 KO mixed chimeras (●; prepared as described in Fig. 1*A*) and 3-Gy-irradiated (WT → KO) chimeric recipients of CD47 KO BMCs (○; prepared as described in Fig. 2*C*). (*B* and *C*) At week 24, WT → CD47 KO chimeras (●; $n = 5$) and age-matched WT (■; $n = 3$) and CD47 KO (□; $n = 3$) controls were injected (i.v.) with CFSE-labeled CD47 KO RBCs, and the clearance/phagocytosis of injected CFSE-labeled CD47 KO RBCs was determined by flow cytometry (*B*) and florescence microscopy (*C*). (*B*) In flow cytometry-based clearance assay, results were normalized with the level of $CFSE^+$ CD47 KO RBCs in blood at 1 h after cell transfer as 100%. (*C*) In microscopic analysis, spleens were harvested 2 h after injection of CFSE-labeled RBCs (green), and frozen spleen sections were stained with anti-F4/80 mAb (red). Engulfment was seen as a yellow event after merging the green-filtered and red-filtered images. (*D*) Clearance of CFSE-labeled CD47 KO RBCs in WT → CD47 KO chimeras that were treated with Cl_2MDP-liposome (Cl_2MDP; $n = 4$) or with liposome-encapsulated saline (Control; $n = 3$). Cl_2MDP-liposomes or liposomes were given 2 days before injection of CFSE-labeled CD47 KO RBCs.

CD47 KO chimeras led to development of mixed hematopoietic chimerism in T, B, and Mac-1^+ cells (Fig. 2 *C* and *D* and data not shown), but all RBCs in these mice were $CD47^{+/+}$ (Fig. 4*A*). To determine whether the loss of CD47 KO RBCs in these chimeras was resulted from phagocytosis, we assessed the ability of these mice to clear exogenously injected CFSE-labeled CD47 KO RBCs. CD47 KO RBCs were rapidly cleared after injection into WT but not CD47 KO mice (Fig. 4*B*). Injected CD47 KO RBCs were also rapidly eliminated in WT → CD47 KO chimeras (Fig. 4*B*). Immunofluorescence staining revealed that large numbers of CD47 KO RBCs were engulfed by $F4/80^+$ macrophages in the spleen in the chimeras and WT mice (Fig. 4*C*). Furthermore, the clearance of injected CD47 KO RBCs was prevented by macrophage depletion with Cl_2MDP-liposomes (Fig. 4*D*), demonstrating that CD47 KO RBCs were eliminated by macrophages in these mice.

To further confirm that macrophages were responsible for the elimination of CD47 KO RBCs in these mice, we followed CD47 KO RBC recovery in chimeric mice after macrophage depletion. The chimeras were treated with four injections of Cl_2MDP-liposomes (0.01 mg/g of body weight) in 5-day intervals. Recovery of CD47 KO RBCs was detected in these mice after injection of Cl_2MDP-liposomes, but the levels of CD47 KO RBCs declined again after withdrawal of the treatment and eventually disappeared from blood circulation (Fig. 5 *A* and *B*). However, macrophage depletion with Cl_2MDP liposomes did not affect other lineages of CD47 KO cells, including Mac-1^+, TCR$\alpha\beta^+$, and $B220^+$ cells in these mice (Fig. 5*C*). The data confirm that

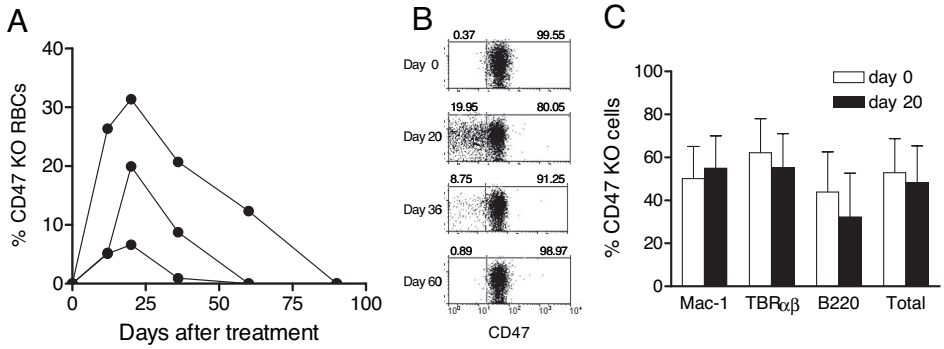

Fig. 5. Recovery of CD47 KO RBCs in long-term (32-week) WT → CD47 KO mixed chimeras after the depletion of phagocytic cells. (*A*) Percentages of CD47 KO RBCs in blood at the indicated times after injection of Cl_2MDP liposomes. Each line represents an individual chimera. (*B*) Flow cytometry profiles showing recovery of CD47 KO RBCs in a chimera after macrophage depletion. (*C*) Percentages (mean ± SDs) of CD47 KO cells in Mac-1^+, TCR$\alpha\beta^+$, and $B220^+$ and total WBCs in mixed chimeras ($n = 3$) before (day 0) and 20 days after first injection of Cl_2MDP-liposomes.

Table 1. Regulation of macrophage function by nonhematopoietic cells in CD47 chimeras

Chimera donor → recipient	Recipient irradiation, Gy	Hematopoietic cells in chimera	Nonhematopoietic cells in chimera	Macrophage tolerance to CD47 KO cells
WT → CD47 KO (Fig. 1)	6 or 3	Mixed	$CD47^-$	Yes
WT → CD47 KO (Fig. 2)	9.5	$CD47^+$	$CD47^-$	Yes
CD47 KO → WT (Fig. 3)	6	Mixed*	$CD47^+$	No

*CD47 KO cells were eventually eliminated by macrophages in these chimeras.

the lack of CD47 KO RBCs in the WT → CD47 KO chimeras was due to phagocytosis rather than a defect in erythropoiesis of CD47 KO stem cells.

Discussion

Tissue macrophages that are differentiated from the same precursor cells exhibit extensive variation in functions depending on their anatomic locations, and the maintenance of tissue-specific characteristics of macrophages is required for an organ to function normally (1, 12, 13). Residential intestinal macrophages display inflammatory anergy characterized by down-regulated expression of innate response and growth factor receptors and reduced production of inflammatory cytokines but remain highly phagocytic (14–16). This unique property of intestinal macrophages is essential to their undiminished capacity to protect the host from foreign pathogens while being less inflammatory in character. Intestinal stromal cells have recently been shown to play a critical role in the generation of intestine-specific macrophages through the production of TGF-β (14). Similarly, lung collectins, surfactant (SP)-A and SP-D have been reported to bind SIRPα on alveolar macrophages to help maintain a non- or antiinflammatory lung environment (17). However, the role of macrophage receptor-specific ligand expression on tissue stromal cells in regulating macrophage function has been overlooked. In this study, we show that nonhematopoietic cells play critical roles in regulation of macrophage function (Table 1). CD47 expression on nonhematopoietic cells confers macrophages with the ability to recognize CD47 as a marker of self and to phagocytose $CD47^{null}$ cells. Likewise, the lack of CD47 on nonhematopoietic cells induces macrophage tolerance to CD47 KO cells. Not only endogenous CD47 KO cells, macrophages in the chimeras where nonhematopoietic cells lacking CD47 also failed to phagocytose exogenously injected splenocytes from untreated CD47 KO mice. Thus, the alterations in macrophages rather than target cells are responsible for the observed macrophage tolerance to CD47 KO cells. However, the altered macrophages in these chimeras retain the ability to phagocytose CD47 KO RBCs (Figs. 4 and 5), suggesting that the mechanisms for preventing phagocytosis of normal cells may differ among different cell types, at least between RBCs and nucleated hematopoietic cells.

MHC class I-specific inhibitory receptors on natural killer (NK) cells prevent the lysis of healthy autologous cells; inasmuch MHC class I-deficient cellular targets are highly susceptible to killing by NK cells (18). However, similar to macrophage tolerance to CD47 KO cells in CD47 KO mice, NK cells from MHC class I-deficient mice showed tolerance to MHC class I-deficient targets (19, 20). Recent studies demonstrate that NK cells acquire functional competence through specific interaction with self-MHC class I molecules, a process termed ''licensing'' (21). This licensing mechanism explains NK cell self-tolerance: (*i*) because only NK cells expressing the inhibitory receptors specific for self-MHC class I can be licensed, all functional NK cells are self-tolerant and (*ii*) unlicensed NK cells, because of the lack of appropriate inhibitory receptors for self-MHC class I or to the lack of MHC class I expression in the host (e.g., MHC class I-deficient mice), are defective in target killing. The cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of the NK cell inhibitory receptors recognizing self-MHC class I molecules are required for licensing (21). Because SIRPα is an ITIM-containing inhibitory receptor, licensing, i.e., SIRPα engagement with CD47 (presumably expressed on nonhematopoietic cells), might also be required for macrophages to acquire effector function. However, functional defect has not been detected in macrophages from mice expressing a mutant SIRPα that lacks most of the cytoplasmic region and does not undergo tyrosine phosphorylation, and macrophages from the mutant mice show rather increased phagocytosis of IgG- or C3bi-opsonized RBCs (22). This suggests that licensing may not be required for macrophages to acquire effector functions, or that licensing through SIRPα-CD47 engagement might be required for macrophages to acquire certain effector functions but not Fcγ receptor- or complement receptor-mediated phagocytosis.

In addition to SIRPα, a number of inhibitory receptors have been reported to inhibit macrophage function, including CD200 receptor (23, 24), paired Ig-like receptor (PIR)-B (25), Ig-like transcript (ILT) 3 (26), and CD33-related receptors (27). It is also possible that macrophages are licensed through multiple inhibitory receptors. In that case, the split macrophage tolerance in CD47 chimeras, i.e., macrophages that are tolerized to CD47 KO nucleated hematopoietic cells remain phagocytic against CD47 KO RBCs, could be because nucleated hematopoietic cells, but not RBCs, express the ligand(s) for the other macrophage receptor(s) that provide inhibitory function in effector responses. Alternatively, the lack of CD47 expression on nonhematopoietic cells may result in compensative up-regulation of other known or unknown inhibitory receptor(s) on macrophages, but the ligands for these receptors are not expressed on RBCs.

Macrophages mediate strong rejection of xenogeneic hematopoietic cells, even in the absence of adaptive immunity (11, 28, 29). The rapid rejection of xenogeneic hematopoietic cells by macrophages greatly impedes the application of mixed chimerism, a means of tolerance induction (30), to xenotransplantation. Our recent studies showed that pig CD47 does not interact with mouse or human SIRPα, and this interspecies incompatibility contributes significantly to phagocytosis of porcine xenogeneic cells by macrophages (31, 32). The current study demonstrates that the lack of CD47 expression on nonhematopoietic cells is required for inducing macrophage tolerance to CD47 KO cells. Thus, the mixed hematopoietic chimerism approach, which has been shown to induce tolerance of T, B, and NK cells (30), is unlikely to induce macrophage tolerance in a xenogeneic combination if the donor CD47 does not interact with the recipient SIRPα. This is consistent with our previous studies where we show that mouse macrophages developing *de novo* in porcine hematopoietic chimeric mice remain phagocytic against porcine hematopoietic cells (11).

Macrophage activation is regulated by the balance between activating and inhibitory signals. Macrophages can be activated by the prophagocytic signaling through activating receptors, such as Fcγ and complement receptors, but their phagocytic activity also largely depends on the signal strength of immune inhibitory

receptors (33). The data presented here demonstrates that the inhibitory receptor signaling that controls macrophage activation in a steady-state condition can be regulated by means of a hematopoietic cell-independent mechanism. This nonhematopoietic cell-mediated regulation of macrophage activity reveals an important mechanism of macrophage tolerance and suggests that modification of local tissue expression of ligands for macrophage receptors may provide an approach for correcting macrophage disorders.

Materials and Methods

Animals. CD47 KO mice on a C57BL/6 (B6) background were generated by back-crossing CD47 KO mice to B6 mice (The Jackson Laboratory, Bar Harbor, ME) (34). CD11c-DTR-Tg [FVB-Tg (Itgax-DTR/EGFP)57Lan/J] mice (35) and normal B6 mice were purchased from The Jackson Laboratory. Protocols involving animals were approved by the Massachusetts General Hospital subcommittee on research animal care.

MACS Separation of Lin$^-$ BMCs. To obtain a Lin$^-$ cell population, BMCs were incubated with biotinylated anti-B220 (B cells), DX5 (NK cells), CD11b (Mac-1, including macrophages), Ter-119 (erythrocytes), CD4 (T cells), and CD8 (T cells) mAbs for 10 min at 4°C. Cells were then incubated with streptavidin beads for 10 min at 4°C, and bead-bound cells were depleted by using the MACS sorting system (Miltenyi Biotec, Auburn, CA) by passing the cells through an LD column. Eluted cells were collected and washed. Depletion was confirmed by FACS analysis, and the percentage of Lin$^+$ cells was $<3\%$ in all Lin$^-$ cell preparations used in this study.

Establishment of CD47 Chimeras and Assessment of Chimerism. WT or CD47 KO B6 recipient mice were conditioned by sublethal (3 or 6 Gy) or lethal (9.5 Gy) whole-body irradiation before i.v. injection of donor BMCs. To prevent initial rejection of CD47 KO donor BMCs by the recipient macrophages, WT recipients were macrophage-depleted by treatment with liposome-encapsulated Cl_2MDP (dichloromethylene diphosphonate or clodronate; 0.02 mg/g of body weight at day -2, and 0.01 mg/g of body weight at days 3, 6, and 11 with respect to BMT). Clodronate was a kind gift of Roche Diagnostics (Mannheim, Germany); liposome-encapsulated clodronate was prepared as described (9). To establish WT $\rightarrow$ CD47 KO chimeras, irradiated CD47 KO recipients were injected with macrophage-free (i.e., 2.5×10^5 Lin$^-$) BMCs from WT donors. The levels of WT and CD47 KO hematopoietic chimerism in various tissues were determined by flow cytometric analysis, in which the cells were stained with anti-CD47 FITC in combination with anti-Mac-1 PE and APC-conjugated mAb against B (B220) or T cells (TCRα/β). All mAbs used were purchased from BD Pharmingen (San Diego, CA).

Cell Clearance Assay. For RBC clearance assay, mice were injected with 5–10 $\times$ 10^7 CFSE-labeled mouse RBCs via tail vein. Five microliters of blood were collected at various times and the percentages of CFSE$^+$ RBCs were determined by flow cytometry. For the splenocyte clearance assay, mice were injected with 3×10^7 CFSE (or PKH26)-labeled mouse splenocytes. Mice were bled at various times after cell injection for preparing WBCs, and the percentages of the fluorescence-labeled cells in the WBCs were determined by flow cytometry. Results are presented as relative percentages of the labeled cells, which are normalized with the levels at 1 h (for RBCs) or 2 h (for splenocytes) after cell transfer as 100%.

Detection of *in Vivo* Phagocytosis by Immunofluorescence Microscopy. Recipient spleens were harvested 2 h after injection of CFSE-labeled CD47 RBCs and stored at -70°C. Frozen sections (8 μm) were prepared, fixed in acetone for 10 min at 4°C, and stained with PE-labeled rat anti-mouse F4/80 (Caltag, Burlingame, CA). After being washed and mounted, slides were viewed under a Nikon Eclipse TE2000 fluorescent microscope (Micro Video Instruments, Avon, MA).

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