Deletion of CD47 from Schwann cells, macrophages and microglia hastens myelin disruption and scavenging in Schwann cells and augments myelin debris phagocytosis in macrophages and microglia

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Research Article

Keywords: CD47, SIRPα, nerve injury, Wallerian degeneration, macrophages, microglia, Schwann cells, phagocytosis, myelin, axon regeneration

Posted Date: January 20th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1268453/v1>

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Abstract

Background: Myelin that surrounds axons breaks in trauma and disease; e.g., PNI and SCI (peripheral nerve and spinal cord injuries) and MS (multiple sclerosis). Resulting myelin debris hinders repair if not effectively scavenged by Schwann cells and macrophages in PNI and by microglia in SCI and MS. We showed previously that myelin debris evades phagocytosis as CD47 on myelin ligates SIRPα (signal regulatory protein-α) on macrophages and microglia, triggering SIRPα to inhibit phagocytosis in phagocytes. Using PNI as a model, we tested the in-vivo significance of SIRPα-dependent phagocytosis inhibition in SIRPα null mice, showing that SIRPα deletion leads to accelerated myelin debris clearance, axon regeneration and recovery of function from PNI. Herein, we tested how deletion of CD47, a SIRPα ligand and a cell surface receptor on Schwann cells and phagocytes, affects recovery from PNI.

Methods: Using CD47 null (CD47^{-/-}) and wild type mice, we studied myelin disruption and debris clearance, axon regeneration and recovery of function from PNI.

Results: As expected from CD47 on myelin acting as a SIRPα ligand that normally triggers SIRPα-dependent phagocytosis inhibition in phagocytes, myelin debris clearance, axon regeneration and function recovery were all faster in CD47^{-/-} mice than in wild type mice. Unexpectedly compared with wild type mice, myelin debris clearance started sooner and CD47-deleted Schwann cells displayed enhanced disruption and scavenging of myelin in CD47^{-/-} mice. Furthermore, CD47-deleted macrophages and CD47-deleted microglia from CD47^{-/-} mice phagocytosed more than CD47-expressing phagocytes from wild type mice.

Conclusions: This study reveals two novel normally occurring CD47-dependent mechanisms that impede myelin debris clearance. First, CD47 expressed on Schwann cells inhibits myelin disruption and debris scavenging in Schwann cells. Second, CD47 expressed on macrophages and microglia inhibits myelin debris phagocytosis in phagocytes. The two add to a third mechanism that we previously documented whereby CD47 on myelin ligates SIRPα on macrophages and microglia, triggering SIRPα-dependent phagocytosis inhibition in phagocytes. Thus, CD47 plays multiple inhibitory roles that combined impede myelin debris clearance, leading to delayed recovery from PNI. Similar inhibitory roles may hinder recovery from other pathologies in which repair depends on efficient phagocytosis (e.g., SCI and MS).

Background

Myelin, a specialized extension of Schwann cells in PNS and oligodendrocytes in CNS (respectively, peripheral and central nervous system), normally surrounds larger diameter axons, enabling transfer and processing of information encoded in electrical signals. Myelin breaks in trauma (e.g., PNI and SCI) and disease (e.g., MS). Resulting myelin debris hinders repair if not rapidly and efficiently cleared. Potentially, Schwann cells and macrophages can clear myelin debris in PNS and microglia in CNS, Schwann cells through autophagy [1,2] and phagocytosis [2,3], and macrophages and microglia through phagocytosis [4-6]. Regrettably, clearance is often deficient, leading to hindered repair.

We have been studying mechanisms that control myelin debris clearance using cultured primary macrophages and microglia in our in-vitro studies and PNI as a model in our in-vivo studies. PNI severs axons at lesion sites, leading to Wallerian degeneration distal to lesion sites [7]. In Wallerian degeneration, axons and myelin break, and mostly recruited macrophages and resident Schwann cells scavenge the debris; e.g., reviewed in [8,9]. To regain function, severed axons must reach their denervated target cells by first crossing the lesion site, then entering and growing throughout the Wallerian degenerating nerve segment. The type of trauma determines if and how many of the severed axons successfully cross the lesion site. In crush injury, the nerve's uninterrupted connective tissue enables efficient crossing. By contrast, the gap between proximal and distal nerve stumps that cut/avulsion injury forms obstructs crossing; e.g., discussed in detail regarding human PNI [10,11]. The nature of Wallerian degeneration that follows all types of nerve injuries determines whether regenerating axons that successfully crossed the lesion site will promptly reach their target cells by growing/regenerating throughout the distal nerve segment. Though possible, less than 50% of patients regain adequate sensory and motor functions [10,11].

We focus in our studies on how Wallerian degeneration affects axon growth/regeneration. Observations in humans and studies in animal models suggest three factors that combined affect axon growth/regeneration. First is the length of the distal nerve segment that may vary from several millimeters to up to over one meter depending on species (e.g. mice versus humans) and site of trauma (e.g., near to versus distant from denervated target cells). Second is the decline with time of the capacity to support axon growth that initially develops in Wallerian degeneration [12,13]. Third is the rate axons grow/regenerate. In humans, axon regeneration through Wallerian degenerating nerve segments was examined after avulsion injuries that required suturing of proximal and distal nerve stumps and after crush injuries that did require surgical intervention. Sensory axons that regenerated initially 2.5 mm/day slowed to 0.5 mm/day and motor axons that initially regenerated 2 mm/day slowed to 1 mm/day [14,15]. Thus, the initial slow rate of axon growth/regeneration slowed down further.

Leading causes of slow axon growth/regeneration in Wallerian degeneration are myelin debris (also referred to as degenerated myelin) and MAG (myelin-associated glycoprotein) [16-19]. Schwann cells and macrophages could alleviate axon growth inhibition by scavenging myelin debris, Schwann cells by autophagy [1,2] and phagocytosis [2,3], and macrophages by phagocytosis [4-6]. Yet, the rate of myelin debris removal is slower than the rate fast regenerating axons grow [20].

Previously, we showed that myelin debris inhibits its own phagocytosis in cultured primary macrophages and microglia through the binding of CD47 on myelin to immune inhibitory receptor SIRPα (also known as CD172a, SHPS-1, p84, gp93 and BIT) on phagocytes, triggering SIRPα to generate “don't eat” signaling [21,22]. In this context, CD47 on myelin functions as a “don't eat me” SIRPα ligand, as previously shown in other systems; e.g., [23,24]. Next, we verified the in-vivo significance of SIRPα-dependent inhibition of myelin debris phagocytosis using PNI as a model [25]. Macrophages from SIRPα^{-/-} mice phagocytosed significantly more than macrophages from wild type mice, and furthermore, myelin debris clearance, axon regeneration and restoration of function were all faster in SIRPα^{-/-} mice than in wild type mice [25].

We designed the current study to verify the in-vivo significance of CD47 on myelin acting as a SIRPα ligand that triggers SIRPα-dependent phagocytosis inhibition in phagocytes [21,22]. In agreement with this notion and our in-vivo findings in SIRPα^{-/-} mice [25], myelin debris clearance, axon regeneration and recovery of function were all faster in CD47^{-/-} mice than in wild type mice. Unexpectedly, the onset of myelin debris clearance in CD47^{-/-} mice preceded that in wild type mice, which was not the case in SIRPα^{-/-} mice compared with wild type mice [25]. This discrepancy led us to look for roles other than acting as a “don’t eat me” SIRPα ligand through which CD47 may affect myelin debris scavenging. Indeed, CD47 (also known as IAP - integrin-associated protein) could play additional roles for two reasons. First, CD47 is a cell membrane receptor that regulates various functions by generating intracellular signaling (e.g., NO production, apoptosis and autophagy) and through lateral association with other cell surface receptors (e.g., integrins) [26-28]. Second, macrophages and Schwann cells express CD47 [21, 25].

Methods

Animals

CD47 null (CD47^{-/-}) and wild type mice colonies were housed at the Hebrew University Faculty of Medicine animal facility as previously reported [21]. Sex- and age-matched 8 to 12 weeks old mice were used in experiments in accordance with the Israeli national research council guide for the care and use of laboratory animals and the approval of the Hebrew University institutional ethic committee.

Surgical procedures

Surgery was performed under anesthesia on one hind limb of wild type and CD47^{-/-} mice as we previously did [25]. Sciatic and saphenous nerves were exposed through small incisions in the overlying skin. Freeze-crush injuries that enable axon regeneration were performed on saphenous nerves using a fine jeweler’s tweezer that was cooled in liquid nitrogen and then applied to nerves for five seconds, taking care to preserve the continuity of the epineurium. Avulsion injuries that do not enable axon regeneration were performed on sciatic nerves by removing a small nerve segment at mid-thigh level. Finally, the skin was sutured and sprayed with antiseptics.

Assessment of the recovery of sensory function after nerve injury

We assessed recovery of sensory function as we previously did [25] using the flexion-withdrawal reflex, withdrawal of hind limbs in response to touching their paws with a blunt pin and von-Frey monofilaments that produce punctate mechanical stimuli delivered mostly by Aδ axons; i.e., pinprick testing. Mice that had their saphenous nerve freeze-crushed were placed on an elevated wire mesh platform until calm, and then, testing of both injured and uninjured limbs was carried out by gently touching paws at areas that saphenous sensory axons normally innervate. Two investigators assessed the recovery of sensory function independently by testing all wild type and CD47^{-/-} mice side by side at one-day intervals after surgery. Each mouse was tested for at least three days after function first returned to verify consistency.

Isolation of primary thioglycollate elicited peritoneal macrophages

Peritoneal cells were harvested in cold DMEM/F12 3 to 4 days after intraperitoneal injection of 1 ml of 3% thioglycollate (Difco, Detroit, MI, USA), as we previously did [29].

Isolation of primary microglia

Microglia were isolated from brains of neonate mice as previously described [5]. In brief, brains were stripped of their meninges, enzymatically dissociated and cells plated on poly-L-lysine coated flasks for 1 week. Non-adherent cells and loosely adhered cells were re-plated for 1 h on bacteriological plates and non-adherent cells washed away, so sorting out cells exhibiting slower kinetics of adherence. The vast majority of adherent cells are microglia judged by morphology [30,31], expression of P2Y₁₂ [32] and positive immunoreactivity to Galectin-3/MAC-2, complement receptor-3 (CR3) and F4/80 in over 95% of them [33,34]. Microglia were maintained and propagated in DMEM/10% HI-FCS and 10% medium conditioned by the L-cell line that produces CSF-1 (American Type Culture Collection, Rockville, VA, USA).

Myelin isolation

The detailed protocol for isolating myelin was previously described [29]. Isolated myelin is “myelin debris” since intact myelin breaks during isolation.

Phagocytosis of myelin debris

Phagocytosis was assayed as previously described; e.g., [29,30]. Macrophages and microglia were plated in 96-well tissue culture plates at a density that minimizes cell-cell contact in the presence of DMEM supplemented by 10% FCS. Non-adherent cells were washed out after 2 h and adherent cells left to rest overnight either in DMEM supplemented by 10% FCS or by 0.1% BSA for experiments carried out, respectively, in the presence or in the absence of serum. Next, macrophages and microglia were washed in DMEM/F12 supplemented, respectively, by 10% FCS or 0.1% BSA, myelin debris added for 30 min, unphagocytosed myelin debris washed out, and levels of phagocytosed myelin debris determined by ELISA.

Detecting and quantifying myelin debris phagocytosis by ELISA

This assay is based on the detection of the myelin-specific protein MBP (myelin basic protein) in phagocytes as previously detailed [29]. Since MBP is unique to myelin and macrophages and microglia do not produce it, MBP levels in phagocyte cytoplasm are proportional to levels of phagocytosed myelin debris. In brief, phagocytes were immediately lysed (50 mM carbonate buffer, pH 10) after myelin debris phagocytosis was completed, lysates transferred to high protein absorbance plates (Thermo Fisher Scientific, Nunc International, USA) in equal volume of coating buffer (0.5 M carbonate buffer pH 9.6). Levels of MBP were determined by ELISA using rat anti-MBP mAb and matching control IgG (Bio-Rad Laboratories Inc., Hercules, USA).

When phagocytosis by macrophages and microglia from wild type mice was compared with phagocytosis by respective phagocytes from CD47^{-/-} mice, phagocytosis by each population was first normalized to the number of respective phagocyte counted in 1-mm² areas at the center of wells. Normalizing phagocytosis to cell number is required since phagocytes from the two mice strains may differ in their adherence properties, thus resulting in different number of adherent cells even when the same number of cells was initially seeded. To this end, phagocytes in replicate plates were fixed, stained and counted. Phagocytosis by phagocytes from CD47^{-/-} mice was calculated as percentage of phagocytosis by phagocytes from wild type mice normalized to 100%.

Quantifying MBP content in nerve tissue

The detailed protocol used to quantify Galectin-3/MAC-2 [35] was previously adopted to quantify MBP in peripheral nerves [25,36]. In brief, nerves were homogenized in 50 mM sodium carbonate buffer pH 9.0 supplemented with protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, USA), protein concentration in cleared extracts was determined using the Bradford assay reagent (Bio-Rad Laboratories Inc., Hercules, USA) and adjusted to 5 µg/mL. Equal volumes (75 µL) of extracts and coating buffer (0.5M carbonate buffer pH 9.6) were incubated overnight at 4⁰C in 96-well high protein absorbance plates (Thermo Fisher Scientific, Nunc International, USA), and levels of MBP determined by ELISA using rat anti-mouse MBP mAb and matching control IgG (Bio-Rad Laboratories Inc., Hercules, USA).

Immune fluorescence confocal microscopy

Nerves were cross-sectioned (8-µm) in a freezing microtome and sections blocked overnight (ON) in 10% FCS in PBS at 4⁰C. To visualize macrophages, sections were incubated ON at 4⁰C in rat anti-mouse monoclonal antibodies (mAbs) M1/70 (Developmental Studies Hybridoma Bank, Iowa City, USA) and 5C6 (American Type Culture Collection, Rockville, USA) that were raised against the αM/CD11b subunit of complement receptor-3 (CR3) that mediates most myelin debris phagocytosis in phagocytes [4-6]. To visualize axons, sections were incubated ON at 4⁰C in rat anti-neurofilament (anti-NF) IgG fraction (Sigma-Aldrich, Israel) diluted 1:5 in 10% FCS in PBS, washed in PBS, fixed in 4% neutral formalin in PBS for 20-min, washed in PBS, incubated for 40-min in FITC-conjugated rabbit anti-rat IgG (Jackson IR laboratories, PA, USA) (diluted 1:500 in 10% FCS in PBS), and finally washed in PBS. Microscopy was carried out in Olympus FluoView FV1000 confocal microscope.

Electron microscopy

Tissues were fixed for 2-hrs in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1M NaCocodylate buffer, washed in 0.1M NaCocodylate buffer, fixed for 1-hr in 1% osmium/1.5% K-ferricyanide in 0.1M NaCocodylate buffer, dehydrated in ethanol, and finally embedded in EPON (all were obtained from Electron Microscopy Sciences, USA). Thin sections were viewed using Tecani-12 transmission electron microscope and photographed by CCD camera MegaView II and software AnalySIS 3.0.

Statistical analysis

The following statistical analyses were carried out using GraphPad Prism software: Gaussian distribution, the parametric unpaired t test and one- and two-way ANOVA, the nonparametric Mann-Whitney test, and the log-rank Mantel-Cox test. Data that passed the normality test were subjected to parametric statistics and those that were too small for testing for normality were subjected to nonparametric statistics.

Results

Myelin debris clearance starts sooner and is faster in CD47^{-/-} mice than in wild type mice

We analyzed the timing of myelin debris clearance and degradation in Wallerian degeneration in the absence of axon regeneration by determining the reduction in nerve-tissue content of myelin-specific protein MBP (myelin basic protein) in nerve segments located distal to but not including lesion sites (Figure 1), as we did previously [25,36]. Intact nerves from CD47^{-/-} and wild type mice displayed similar MBP content, indicating similar myelin content. Compared with intact nerves, MBP content decreased significantly as of day 2 after surgery in CD47^{-/-} mice but only as of day 4 after surgery in wild type mice. Overall, MBP content decreased significantly more in CD47^{-/-} mice than in wild type mice on days 2, 3, and 4 after surgery. The advanced clearance of myelin in CD47^{-/-} mice was also evident on days 5 and 7 after surgery though not statistically significant. Thus, significant clearance and degradation of myelin debris started sooner and continued faster in CD47^{-/-} mice compared with wild type mice.

Sensory function recovers faster in CD47^{-/-} mice than in wild type mice

For studying how Wallerian degeneration affects the growth/regeneration of severed axons and thereby recovery of function from PNI it is advantageous to follow as many regenerating axons as possible. For that purpose, we inflicted freeze-crush injuries to sensory saphenous nerves. This type of injury severs all axons while preserving the continuity of the nerve connective tissue, enabling a large proportion of regenerating axons to cross the lesion site, then successfully enter the distal Wallerian degenerating nerve segment.

To test the recovery of sensory function, we used the flexor-withdrawal reflex, hind limb withdrawal in response to gently touching the paw. The saphenous and sciatic nerves provide sensory innervation to the hind limb paw and the sciatic nerve further supplies motor innervation to hind limb muscles. We freeze-crushed saphenous nerves at an average distance of 14 mm from paws. At the same time and same limb, we resected a segment of the sciatic nerve at mid-thigh level to prevent axon regeneration but spare hip joint flexion and thereby limb withdrawal. Hence, reflex recovery depended solely on successful regeneration and skin reinnervation by regenerating saphenous sensory axons. We operated on and tested wild type and CD47^{-/-} mice side by side at one-day intervals after surgery (Figure 2). The reflex disappeared for at least two days after surgery, confirming successful sensory denervation of paws. In CD47^{-/-} mice, the reflex returned in 17% of mice on day 3, median recovery was on day 5 and all mice had regained the reflex by day 7 after surgery. In wild type mice, the reflex returned in 4% of mice on day 5, median recovery was on day 7 and all mice regained the reflex by day 10 after surgery. Remarkably, on

day 5 after surgery, 74% of CD47^{-/-} mice regained the reflex whereas only 4% of WT mice did so, reflecting 14.8 fold higher recovery rate in CD47^{-/-} mice at that time. Thus, sensory function recovered significantly faster in CD47^{-/-} mice than in wild type mice.

Severed axons regenerate faster in CD47^{-/-} mice than in wild type mice

The earlier recovery of sensory function in CD47^{-/-} than in wild type mice (Figure 2) resulted most likely from faster growth/regeneration of their severed saphenous nerve sensory axons. To verify that this is the case, we visualized axons by positive immunoreactivity to NF (neurofilaments) in intact and Wallerian degenerating saphenous nerves sampled 10 to 12 mm distal to lesion sites (Figure 3A). NF immunoreactivity decreased substantially in both CD47^{-/-} and wild type mice at 2.5 days after surgery, indicating loss of axons due to rapid degeneration. NF immunoreactivity increased markedly in CD47^{-/-} mice but less in wild type mice at 4.5 days after surgery, indicating quicker appearance of newly regenerating axons at the sampling site in CD47^{-/-} mice than in wild type mice. Indeed, at 4.5 days after surgery, the number of NF positively marked axons was significantly 2.3 fold higher in CD47^{-/-} than in wild type mice (Figure 3B). These observations are in good agreement with the loss of sensory function in all mice for the first two days after surgery and functional recovery in 17% of CD47^{-/-} mice but in none of wild type mice on day 3 after surgery (Figure 2). Thus, sensory saphenous nerve axons grew/regenerated faster through Wallerian degenerating nerves in CD47^{-/-} mice than in wild type mice.

Hastened and augmented in-vivo disruption and debris scavenging of myelin in CD47-deleted Schwann cells compared with CD47-expressing Schwann cells

The clearance of myelin debris was faster in CD47^{-/-} mice than in wild type mice, and furthermore, the onset of clearance in CD47^{-/-} mice preceded that in wild type mice by 2 days (Figure 1). We expected faster but not sooner onset of clearance based on the notion that deleting CD47 from myelin omits myelin's CD47 role as a SIRPα ligand that normally triggers SIRPα-dependent phagocytosis inhibition in macrophages [21,25]. Evidently, that was not the case. We searched, therefore, for other mechanisms through which CD47 may affect myelin debris scavenging. In this regard, CD47 deletion from Schwann cells and/or macrophages should be considered since the two cell types scavenge myelin debris in Wallerian degeneration and both express CD47 [21,25].

We focused first on Schwann cells, reasoning that disruption of the normal compact lamellar architecture of their myelin should precede myelin debris scavenging whether by Schwann cells or macrophages. If so, the expectation is that myelin disruption will start sooner and/or be faster in CD47^{-/-} mice than in wild type mice. To address this possibility, we studied myelin ultrastructure in Wallerian degenerating sciatic nerves in the absence of axon regeneration. We sampled injured nerves at a distance of 5 to 6 mm distal to but not including lesion sites on days 2 to 2.5 after surgery. This timing corresponds with clearance onset in CD47^{-/-} mice but precedes clearance onset in wild type mice (Figure 1). We observed a wide range of structural changes from normal in myelin and further detected myelin debris in Schwann cells' cytoplasm in the two mice strains, but at higher frequencies in CD47^{-/-} mice than in wild type mice (Figures 4 and 5). Normally, flat myelin sheaths coil around axons forming tightly laminated spiral

windings round them (Figure 4A). In Wallerian degeneration, sections of myelin spirals delaminate and further become unwound exposing spaces between layers (Figure 4B, C and D). Unwound sections of myelin sheaths form small coils of which some remain attached and some become detached from the large spirals (Figure 4E) and other become internalized into Schwann cells' cytoplasm (Figure 4F). Percent of Schwann cells that presented abnormal structure of their myelin (i.e., myelin disruption) was significantly 2.7 fold higher in CD47^{-/-} mice than in wild type mice (Figure 5A) and percent of Schwann cells that contained myelin debris in their cytoplasm was significantly 2.4 fold higher in CD47^{-/-} mice than in wild type mice (Figure 5B). Thus, deletion of CD47 from Schwann cells hastened and augmented myelin disruption and myelin debris scavenging in CD47^{-/-} mice's CD47-deleted Schwann cells.

Schwann cells scavenge myelin debris through autophagy and phagocytosis. Thus, CD47 deletion could affect either both or one of the two. Morphology could help distinguishing between autophagy and phagocytosis since myelin debris should be present within double-membrane autophagosomes in autophagy and within single-membrane phagosomes in phagocytosis. However, we find it difficult to distinguish between the two with great certainty at all times, which is mandatory for quantitation, due to the lamellar organization of myelin. Nonetheless, our findings suggest that CD47 that Schwann cells normally express impedes the disruption of their myelin and Schwann cells' ability to clear/scavenge myelin-debris in Wallerian degeneration.

Comparable numbers of CR3 expressing phagocytes/macrophages in Wallerian degeneration in CD47^{-/-} and wild type mice

Recent studies suggest that recruited monocyte-derived macrophages outnumber resident macrophages during the first 7 days of Wallerian degeneration, and that mostly recruited macrophages clear myelin debris by phagocytosis [37-39]. Since macrophages normally express CD47 [21], deletion of CD47 from them could accelerate and increase their recruitment and/or augment their phagocytic capacity.

We addressed the issue of accelerated and increased recruitment by quantifying the number of cells that express CR3 (complement receptor-3; also known as MAC-1) that mediates much of the phagocytosis of myelin debris in macrophages and microglia, which we previously documented [4-6]. For this purpose, we sampled intact and Wallerian degenerating saphenous nerves 10 to 12 mm distal to lesion sites, visualizing CR3-expressing (CR3⁺) cells by detecting immunoreactivity to CD11b/αM subunit of CR3. CR3 immunoreactivity was infrequent in intact nerves in the two mice strains, which agrees with previously reported rare detection of 1.2 macrophages/100 μm² in intact nerves [40]. The number of CR3⁺ cells increased progressively to similar levels in the two mice strains from day 2 to day 7 after surgery (Figure 6). This finding agrees with our previous observations that the number of cells expressing the macrophage specific F4/80 antigen increased progressively from 2.5 to 7 days after PNI [3] and with recent findings by others [37-39]. Noteworthy, CR3⁺ cells could be both macrophages and neutrophils [41]. However, most are macrophages since macrophages outnumber neutrophils through the entire period of myelin debris clearance. Taken altogether, the majority of CR3⁺ cells that we detected are most likely recruited monocyte-derived macrophages. The comparable number of CR3⁺ cells/macrophages in

CD47^{-/-} and wild type mice during the first 7 days of Wallerian degeneration suggests that it is unlikely that the earlier onset of myelin debris clearance (Figure 1) resulted from differences in macrophage number between the two mice strains.

Augmented phagocytic capacity in CD47-deleted macrophages and microglia from CD47^{-/-} mice compared with that in CD47-expressing phagocytes from wild type mice

We previously showed that both CD47 and SIRPα are expressed on macrophages and microglia whereas CD47 but not SIRPα are present on Schwann cells and myelin [21,25]. This raises the possibility that CD47 deletion from macrophages and microglia in CD47^{-/-} mice could have altered their phagocytic capacity. We addressed this possibility by studying phagocytosis of myelin debris from wild type and CD47^{-/-} mice (WT and CD47^{-/-} myelin) in cultured macrophages and microglia from wild type and CD47^{-/-} mice (WT and CD47^{-/-} phagocytes) in the presence and in the absence of serum (Figure 7). This experimental paradigm enables testing how deletion of CD47 from phagocytes affects their phagocytic capacity in the absence and in the presence of SIRPα-dependent phagocytosis inhibition. We reached this paradigm based on our previous findings that CD47 on myelin and serum, each by their own and combined, trigger SIRPα-dependent phagocytosis in wild type phagocytes (Figure 7A, inhibitions “a” and “b”) and [21].

In the absence of serum, CD47^{-/-} macrophages phagocytosed significantly 2.2 fold more CD47^{-/-} myelin debris than WT macrophages (Figure 7B), indicating greater phagocytic capacity in CD47^{-/-} than in WT macrophages in the absence of SIRPα-dependent inhibition that CD47 on myelin and serum normally induce (Figure 7A, inhibitions “a” and “b” are not functioning). In the absence of serum, CD47^{-/-} macrophages phagocytosed significantly 1.7 fold more WT myelin debris than WT macrophages (Figure 7C), indicating greater phagocytic capacity in CD47^{-/-} than in WT macrophages in the presence of SIRPα-dependent inhibition that CD47 on myelin induces in the absence of serum (Figure 7A, inhibition “a” is functioning and inhibition “b” is not). Next in the presence of serum, CD47^{-/-} microglia phagocytosed significantly 2.3 fold more WT myelin debris than WT microglia (Figure 7D), indicating greater phagocytic capacity in CD47^{-/-} than in WT microglia in the presence of SIRPα-dependent inhibition that both CD47 on myelin and serum induce (Figure 7A, inhibitions “a” and “b” are functioning). Taken altogether, CD47-deleted phagocytes displayed augmented phagocytosis in the absence and in the presence of SIRPα-dependent phagocytosis inhibition. This suggests that CD47 and SIRPα that macrophages and microglia normally express inhibit phagocytosis and inhibitions by the two receptors are, at least in part, independent of one another and additive.

Discussion

This study reveals two novel normally occurring CD47-dependent mechanisms that impede myelin debris clearance. First, CD47 that Schwann cells express inhibits myelin disruption and myelin debris scavenging in Schwann cells. Second, CD47 that macrophages and microglia express inhibits myelin debris phagocytosis in phagocytes. The two add to a third mechanism that we previously documented

whereby CD47 on myelin ligates SIRPα on macrophages and microglia, triggering SIRPα-dependent phagocytosis inhibition in phagocytes [21,22,25]. Thus, CD47 plays multiple inhibitory roles that combined impede myelin disruption and debris clearance in injury-induced Wallerian degeneration. The resulting delayed clearance of myelin debris leads to slow axon growth/regeneration and protracted recovery of function. Similar CD47-dependent phagocytosis inhibition mechanisms may also contribute to protracted repair in other pathologies in which efficient phagocytosis is critical to repair (e.g., phagocytosis of myelin debris in MS and SCI, and phagocytosis of tumor cells).

Myelin debris clearance in CD47^{-/-} mice preceded that in wild type mice by two days (Figure 1). We suggest that the earlier onset of myelin debris clearance in CD47^{-/-} mice is mostly due to omitting a mechanism by which CD47 normally delays myelin disruption and myelin debris scavenging in Schwann cells. We base this suggestion on our ultrastructural studies on days 2 to 2.5 after injury, a time window at which significant myelin debris scavenging had already begun in CD47^{-/-} but not yet in wild type mice (Figure 1). At that time, myelin disruption and debris internalization into Schwann cells' cytoplasm were already in progress and significantly greater in CD47^{-/-} than in wild type mice, thus greater in CD47-deleted than in wild type CD47-expressing Schwann cells (Figures 4 and 5). The molecular mechanism by which CD47 delays myelin disruption and debris scavenging needs verification. We suggest nonetheless that it may relate, at least in part, to CD47's established role as a cell surface receptor that inhibits autophagy [42] since Schwann cells scavenge myelin debris through autophagy [1,2], albeit also by phagocytosis [2,3].

We suggest that CD47-deleted macrophages in CD47^{-/-} mice contribute little if any to the two day earlier onset of myelin debris clearance but contribute significantly to faster clearance at later stages of Wallerian degeneration. We base this suggestion on the understanding that the phagocytic capacity of single macrophages and the total number of macrophages that are present in Wallerian degeneration at any given time together determine how much myelin debris a given population of macrophages clears. The phagocytic capacity of macrophages in CD47^{-/-} mice exceeds that in wild type mice due to the deletion of CD47 from both macrophages and myelin. CD47-deleted macrophages phagocytosed more than wild type CD47-expressing macrophages (Figure 7), very likely due to the exclusion of a mechanism by which CD47 expressed on macrophages inhibits phagocytosis (discussed below). Additionally, macrophages phagocytosed more CD47-deleted myelin than wild type CD47-expressing myelin by excluding the mechanism by which CD47 expressed on myelin triggers SIRPα-dependent phagocytosis inhibition in macrophages [21,22,25]. It is unlikely that this overall increase in macrophages' phagocytic capacity could contribute much to the two day earlier onset of myelin debris clearance in CD47^{-/-} mice since only few macrophages are present during the first two days of Wallerian degeneration (Figure 6). By contrast, it is most likely that the increased number of CD47-deleted macrophages at later stages of Wallerian degeneration (Figure 6) enables the entire growing population of CD47-deleted macrophages to fully implement their increased phagocytic capacity and so significantly contribute to faster clearance of myelin debris in CD47^{-/-} mice.

CD47-deleted microglia from CD47^{-/-} mice phagocytosed more myelin debris than wild type CD47-expressing microglia from wild type mice (Figure 7C), very likely due to the exclusion of a mechanism by which CD47 expressed on microglia inhibits phagocytosis (discussed below). If so, microglia and macrophages share two distinct CD47-dependent mechanisms that normally inhibit phagocytosis in-vivo. First, CD47 expressed on myelin acting as a SIRPα ligand triggers SIRPα to inhibit phagocytosis in the two phagocytes [21,22,25]. Second, CD47 expressed on phagocytes acting as a cell surface receptor inhibits phagocytosis in them. The exact molecular mechanism by which CD47 on macrophages and microglia inhibits phagocytosis needs verification. We suggest nonetheless that CD47 could inhibit phagocytosis, at least in part, by acting as cell surface receptor that upon activation lowers cAMP levels. We base this suggestion on our previous findings that inhibiting cAMP signaling through PKA reduces myelin debris phagocytosis in macrophages and microglia [43] and findings by others that CD47 is Gi-coupled and upon activation CD47 reduces cAMP levels and consequently signaling through PKA [44-46]. A potential ligand that could activate CD47 is thrombospondin-1 that macrophages and microglia amongst other cells produce and secrete [47,48]. Our findings in this study that CD47 that macrophages and microglia express inhibits phagocytosis in both the presence and the absence of SIRPα-dependent phagocytosis inhibition (Figure 7) further suggest that phagocytosis inhibitions by CD47 and SIRPα that the two phagocytes express are, at least in part, independent of one another and additive.

The faster removal of axon growth-inhibitory myelin debris in CD47^{-/-} mice accounts most likely for faster axon growth/regeneration and so to facilitated recovery of function. Our current findings and observations by others suggest this. First, we show in this study that the slower removal of myelin debris is associated with slower axon growth/regeneration and delayed recovery of function in wild type CD47-expressing mice compared with CD47^{-/-} mice. Second, live in-vivo imaging in wild type mice shows that myelin debris slows axon growth/regeneration [20]. Third, the axon growth-inhibitory properties of myelin and MAG are well-documented [16-19]. Thus, CD47 normally prevents severed axons from fully implementing their regenerative potential by impeding myelin debris clearance through multiple mechanisms.

A point of consideration is whether genetic deletion of CD47 in CD47^{-/-} mice could lead to accelerated axon growth/regeneration by affecting neurons directly. Observations made in human neuroblastoma cells and mouse primary cortical neurons show that transcription factor α-Pal/NRF-1 acting through CD47/IAP promotes and reduced expression of the two impairs neurite outgrowth [49]. Moreover, findings in cultured hippocampal neurons from CD47^{-/-} and wild type mice show that CD47 expression promotes and CD47 deletion impairs neurite outgrowth [50]. Thus, it is unlikely that genetic deletion of CD47 from neurons contributed to accelerated axon growth/regeneration in our current study.

Conclusions

CD47 plays three inhibitory roles that combined impede myelin debris clearance in Wallerian degeneration, leading to slow axon growth/regeneration and retarded recovery from PNI. First, CD47 expressed on Schwann cells inhibits myelin disruption and scavenging in Schwann cells. Second, CD47

expressed on macrophages (and microglia) inhibits phagocytosis in phagocytes. Third, CD47 on myelin triggers SIRP α on macrophages (and microglia) to inhibit phagocytosis in phagocytes. It is highly likely that similar mechanisms may also hinder repair in other neurodegenerative pathologies in which myelin breaks. For example, phagocytosis inhibitions through CD47 that microglia and macrophages express and through CD47 on myelin ligating SIRP α on phagocytes may both contribute to delayed myelin debris clearance in MS. Furthermore, these two inhibitory mechanisms may inhibit phagocytosis in SIRP α - and CD47-expressing wild type phagocytes (e.g., microglia and macrophages) of any cellular target on which CD47 is expressed (e.g., red blood cells [23], platelets [51] and tumor cells [52,53]).

Abbreviations

SIRP α (signal regulatory protein- α), MAG (myelin-associated glycoprotein), MCSF (colony stimulating factors), MBP (myelin basic protein), CR3 (complement receptor-3), NF (neurofilaments), PNS (peripheral nervous system), CNS (central nervous system), PNI (peripheral nerve injury), SCI (spinal cord injury), MS (multiple sclerosis).

Declarations

Ethics

Mice were used in experiments in accordance with the Israeli national research council guide for the care and use of laboratory animals and the approval of the Hebrew University institutional ethic committee.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This project was supported by grant number 1658/14 from the Israel Science Foundation, and The Charles Wolfson Charitable Trust.

Authors' contributions MG, GE, FR and MT carried out experiments and contributed to the writing of the manuscript. SR designed and supervised experiments, and wrote the manuscript.

Acknowledgements

We thank Dr. Sara Leven for reviewing and commenting on the manuscript.

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Figures

Figure 1

Earlier and faster clearance of myelin debris in CD47^{-/-} mice than in wild type mice. Sciatic nerve segments undergoing Wallerian degeneration were removed from wild type (WT) and CD47^{-/-} mice at the indicated days after surgery, immediately lysed and protein content in lysates quantified. Levels of myelin-specific MBP (Myelin/MBP) in lysate samples of equal protein content were quantified using ELISA. Levels of Myelin/MBP are presented as percentage of levels in intact nerves (time 0) normalized to 100%. Box and whisker plot of Myelin/MBP levels in 4 to 17 different nerves at the indicated days after surgery are given. The line represents the median, the box outlines the 25% to 75% range, and whiskers extend to the highest and lowest observations. Significance of difference of WT mice from SIRP α ^{-/-} mice at the indicated days after surgery is ^p<0.05 and ***p<0.001, by two-way ANOVA and the Bonferroni multiple comparisons posttest. Significance of difference between levels of Myelin/MBP in intact nerves

(day 0) and those at the indicated days after surgery is * $p < 0.05$, ** $p < 0.001$ and *** $p < 0.0001$, by one-way ANOVA and the Dunnett posttest calculated for each mouse strain separately.

Figure 2

Sensory function recovers faster in CD47^{-/-} mice than in wild type mice. The flexor-withdrawal reflex recovery curves display the cumulative percentage of CD47^{-/-} and wild type (WT) mice that regained sensory function at each of the indicated days after surgery. Findings from male and female mice were combined since the two genders did not differ in recovery time. Overall, 22 WT mice and 23 CD47^{-/-} mice were tested. Significance of difference of WT mice from SIRPα^{-/-} mice is $p < 0.0001$, by the log-rank Mantel-Cox test.

Figure 3

Severed axons regenerate faster in CD47^{-/-} mice than in wild type mice.

(A) Axons from wild type mice (WT; a, b and c) and from CD47^{-/-} mice (d, e and f) were visualized in cryostat sections by immunofluorescence confocal microscopy using anti-neurofilament Abs (anti-NF; red). Intact and freeze-crushed saphenous nerves were sampled 10 to 12 mm distal to lesion sites at the indicated days after surgery. At 2.5 days (2.5d) after surgery, NF immunoreactivity decreased in both WT mice (b) and CD47^{-/-} mice (e). At 4.5 days (4.5d) after surgery, NF immunoreactivity increased markedly more in CD47^{-/-} mice (f) than in wild type mice (c). Bars: 50 μm . (B) Counts of NF labeled axons (NF-axons) sampled 10 to 12 mm distal to lesion sites at 4.5 days after surgery (e.g., c and f). Box and whisker plot of NF-axons in 10 sections from 4 different WT mice nerves and 11 sections from 3 different CD47^{-/-} mice nerves. The line represents the median, the box outlines the 25% to 75% range, and whiskers extend to highest and lowest observations. Significance of difference between WT and CD47^{-/-} mice is ***p<0.0001, by unpaired t test.

Figure 4

In-vivo myelin disruption and debris scavenging in CD47-deleted Schwann cells in CD47^{-/-} mice.

Micrographs of cross sections from (A) intact nerves and (B through F) Wallerian degenerating nerves taken 5 to 6 mm distal to but not including lesion sites on days 2 to 2.5 after surgery. (A) In intact nerves, flat myelin sheaths form tightly laminated spirals around intact axons. (B through F) In Wallerian degeneration, myelin spirals delaminate and unwind (B, C and D), forming small myelin coils of which some remain attached and some detach from large myelin spirals (E), and some Schwann cells internalize myelin debris (F). Notably, microtubules and neurofilaments enrich the cytoplasm of intact

axons (A), disrupted amorphous axonal cytoplasm embeds unwounded sections of myelin spirals (B, C, D and E), and numerous mitochondria enrich Schwann cells' cytoplasm that embeds myelin debris (F).
 Bras: A, C, E and F 1 μm ; B 2 μm ; D 0.5 μm .

Figure 5

Myelin disruption and scavenging in CD47-deleted Schwann cells exceed disruption and scavenging in CD47-expressing Schwann cells. Schwann cells were randomly sampled in micrographs of cross sections of Wallerian degenerating nerves from wild type (WT) and CD47^{-/-} mice taken on days 2 to 2.5 after surgery. Nerves were sampled 5 to 6 mm distal to but not including lesion sites (e.g., Figure 4 B through F). Box and whisker plots of (A) percent of Schwann cells presenting disrupted myelin (e.g., Figure 4B, C and D) and (B) percent of Schwann cells that contain myelin debris in their cytoplasm (e.g., Figure 4F). In (A), total of 673 WT and 874 CD47^{-/-} Schwann cells were sampled, respectively, in 4 WT and 6 CD47^{-/-} injured nerves. In (B), total of 673 WT and 874 CD47^{-/-} Schwann cells were sampled, respectively, in 5 WT and 6 CD47^{-/-} injured nerves. The line represents the median, the box outlines the 25% to 75% range, and whiskers extend to the highest and lowest observations. Significance of difference between WT and CD47^{-/-} mice is * $p < 0.05$ and ** $p < 0.01$, by Mann Whitney test.

Figure 6

Comparable numbers of CR3 expressing cells/macrophages in Wallerian degeneration in CD47^{-/-} and wild type mice. (A) Cells/macrophages were visualized in cryostat sections from Wallerian degenerating sensory saphenous nerves of wild type mice (WT; a, b, c and d) and CD47^{-/-} mice (e, f, g and h) by immunofluorescence confocal microscopy using mAbs against αM/CD11b subunit of CR3 (red). Wallerian degenerating nerves were cross-sectioned 10 to 12 mm distal to lesion sites on days 1, 2 and 3 (1d, 2d and 3d) after surgery. In intact nerves, CR3 immunoreactivity was barely detected in both WT mice (a) and CD47^{-/-} mice (e). After injury, CR3 immunoreactivity increased progressively in both WT mice (b, c and d) and CD47^{-/-} mice (f, g and h). Bars: 50 μm. (B) Counts of CR3 labeled cells/macrophages (CR3⁺) in micrographs of cross sections from intact (time 0) and Wallerian degenerating saphenous nerves (such as shown in A) at the indicated days after surgery. Box and whisker plots CR3⁺ expressing cells/macrophages in 6 to 18 sections from 4 different WT mice nerves and 4 different CD47^{-/-} mice nerves that were sampled at the indicated days after surgery. The line represents the median, the box outlines the 25% to 75% range, and whiskers extend to highest and lowest observations. Significance of difference from 0 days (intact) is *p<0.05, **p<0.01 and ***p<0.0001, by one-way ANOVA and the Dunnett posttest, calculated for each mice strain separately.

Figure 7

Greater phagocytic capacity in CD47-deleted than in CD47-expressing macrophages and microglia. (A) Activation ($\rightarrow$) and inhibition ($\perp$) of myelin debris phagocytosis - a schematic view. Wild type myelin (WT myelin) from wild type mice, either or not opsonized by complement protein C3bi ($\pm$ C3bi), binds and activates CR3 that mediates much of the phagocytosis of myelin debris in context of traumatic injury [4-6]. CD47 on WT myelin (a) and serum (b) trigger each SIRP α -dependent phagocytosis inhibition in wild type macrophages and microglia (WT phagocytes). Tested hypothesis (c): CD47 on phagocytes triggers phagocytosis inhibition. A potential ligand (?) that could activate CD47 is thrombospondin-1 (TSP1; see Discussion). (B and C) Phagocytosis in serum free medium of myelin debris from (B) CD47^{-/-} mice (CD47^{-/-} myelin) and from (C) wild type mice (WT myelin) by WT and CD47^{-/-} macrophages from WT and CD47^{-/-} mice. (D) Phagocytosis in serum containing medium of WT myelin by WT and CD47^{-/-} microglia from WT and CD47^{-/-} mice. Macrophages and microglia were plated at low density, exposed to myelin debris for 30 min and levels of phagocytosed myelin debris quantified by ELISA. In each experimental paradigm, phagocytosis levels in CD47^{-/-} macrophages and CD47^{-/-} microglia are presented as percentage of phagocytosis levels in the respective WT phagocyte normalized to 100%. Box and whisker

plots of (B) 24 replicates in 7 experiments, (C) 18 replicated in 6 experiments, and (D) 12 replicates in 3 experiments are given. The line represents the median, the box outlines the 25 to 75% range, and whiskers extend to the highest and lowest observations. Significance of difference between WT and CD47^{-/-} phagocytes is *** $p < 0.001$, by unpaired t test.