Intracellular phospholipase A$_2$ group IVA and group VIA play important roles in Wallerian degeneration and axon regeneration after peripheral nerve injury

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We provide evidence that two members of the intracellular phospholipase A$_2$ family, namely calcium-dependent group IVA (cPLA$_2$ GIVA) and calcium-independent group VIA (iPLA$_2$ GVIA) may play important roles in Wallerian degeneration in the mouse sciatic nerve. We assessed the roles of these PLA$_2$s in cPLA$_2$ GIVA$^{-/-}$ mice, and mice treated with a selective inhibitor of iPLA$_2$ GVIA (FKGK11). Additionally, the effects of both these PLA$_2$s were assessed by treating cPLA$_2$ GIVA$^{-/-}$ mice with the iPLA$_2$ inhibitor. Our data suggest that iPLA$_2$ GVIA may play more of a role in the early stages of myelin breakdown, while cPLA$_2$ GIVA may play a greater role in myelin clearance by macrophages. Our results also show that the delayed myelin clearance and Wallerian degeneration after sciatic nerve crush injury in mice lacking cPLA$_2$ and iPLA$_2$ activities is accompanied by a delay in axon regeneration, target re-innervation and functional recovery. These results indicate that the intracellular PLA$_2$s (cPLA$_2$ GIVA and iPLA$_2$ GVIA) contribute significantly to various aspects of Wallerian degeneration in injured peripheral nerves, which is then essential for successful axon regeneration. This work has implications for injury responses and recovery after peripheral nerve injuries in humans, as well as for understanding the slow clearance of myelin after CNS injury and its potential consequences for axon regeneration.

Introduction

Peripheral nerve injury leads to rapid axonal degeneration and removal of degenerating myelin sheaths distal to the site of lesion (Waller, 1850; George and Griffin, 1994). This process, known as Wallerian degeneration, includes rapid axonal degeneration and myelin clearance. Axonal degeneration occurs within the first 48 h after injury (Beirowski *et al.*, 2005) and appears to require calcium-mediated proteolytic activity (Schlaepfer and Bunge, 1973; George *et al.*, 1995). Myelin degradation is a relatively slower process (lasting several days) that is not yet fully understood but appears to require the contribution of both

Schwann cells and macrophages (Stoll *et al.*, 2002). Peripheral nerve myelin, which contains axon growth inhibitory molecules, has been shown using *in vitro* neurite growth assays to be as inhibitory as CNS myelin (David *et al.*, 1995). The clearance of myelin, which contains these axon growth inhibitors, appears to be essential for successful axon regeneration after peripheral nerve crush injury (Schafer *et al.*, 1996). Furthermore, the slow clearance of myelin in the CNS after injury may contribute to the failure of axon regeneration (David and Lacroix, 2003). Therefore, understanding the molecular control of myelin clearance after peripheral nerve injury may provide

insights into promoting more rapid myelin clearance and axon regeneration in the CNS.

A number of factors, such as chemokines and cytokines, have been reported to influence macrophage responses in Wallerian degeneration (Carroll and Frohnert, 1998; Toews et al., 1998; Shamash et al., 2002; Stoll et al., 2002; Tofaris et al., 2002; Perrin et al., 2005). In this article we examined the role of phospholipase A$_2$ (PLA$_2$) enzymes in different stages of Wallerian degeneration. PLA$_2$ enzymes hydrolyze the ester bond at the *sn*-2 position of phospholipids to generate a free fatty acid [e.g. arachidonic acid (AA)], and a lysophospholipid [e.g. lysophosphatidylcholine (LPC)]. There are several forms of mammalian PLA$_2$s that are grouped into secreted (sPLA$_2$) and intracellular PLA$_2$s that include calcium-dependant (cPLA$_2$) and calcium-independent (iPLA$_2$) forms (Murakami et al., 1997; Six and Dennis, 2000; Schaloske and Dennis, 2006). PLA$_2$s play a normal physiological role in phospholipid metabolism, membrane turnover, host defence, and signal transduction (Dennis, 1997; Brown et al., 2003). However, they have also been implicated in inflammation in a variety of tissues and organs, including the nervous system (Murakami, 2004; Farooqui and Horrocks, 2006; Yaksh et al., 2006). We showed previously that sPLA$_2$ group IIA (GIIA) and cPLA$_2$ GIVA are expressed by Schwann cells and macrophages after sciatic nerve injury (De et al., 2003). In addition, treatment of C57BL/6 mice, which have a naturally occurring null mutation of sPLA$_2$ GIIA (Kennedy et al., 1995), with a chemical inhibitor (AACOCF$_3$) that blocks all members of the intracellular PLA$_2$ family (cPLA$_2$ and iPLA$_2$) leads to reduced clearance of myelin debris (De et al., 2003). As this inhibitor was infused into the cut segment of the nerve with an osmotic pump, the effects of this non-specific inhibitor could only be assessed on Wallerian degeneration for up to 7 days. The differential roles, if any, of the two main intracellular PLA$_2$s (cPLA$_2$ GIVA and iPLA$_2$ GVIA) on Wallerian degeneration and axon regeneration is still not known.

We used cPLA$_2$ GIVA null mice and systemic treatment with a novel selective iPLA$_2$ GVIA inhibitor to dissect out the roles of cPLA$_2$ GIVA and iPLA$_2$ GVIA and their combined effects in various stages of Wallerian degeneration and subsequent axon regeneration in the mouse sciatic nerve for up to 21 days. We show that cPLA$_2$ GIVA and iPLA$_2$ GVIA appear to play differential roles in different aspects of myelin breakdown and clearance and that both are required for efficient Wallerian degeneration. Lack of activity of one or both of these PLA$_2$s leads to delayed axon regeneration, target re-innervation and functional recovery.

Materials and Methods
Surgery
All surgical procedures were approved by the McGill University Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care.

The C57BL/6 mouse strain has a naturally occurring null mutation of one of the major forms of inflammatory sPLA$_2$ (group IIA) (Kennedy et al., 1995). This mouse strain was therefore used to assess the role of the intracellular PLA$_2$s (cPLA$_2$ and iPLA$_2$) in Wallerian degeneration using cPLA$_2$ null mice on the C57BL/6 background, and treating wild-type C57BL/6 mice with an iPLA$_2$ inhibitor (see below).

Adult female mice (10–12 weeks old) were anesthetized with ketamine: xylazine: acepromazine (50:5:1 mg/kg), and the right sciatic nerve exposed and crushed with liquid nitrogen-cooled fine forceps (Dumont no 5) for 30 s. The lesion site was about 45 mm from the tip of the third digit. The crush site was labelled with one 10-0 suture through the epineurium of the peroneal branch used for analysis.

cPLA$_2$ GIVA$^{-/-}$ mice on the C57BL/6 background and wild-type littermates generated by breeding heterozygotes were used to study the role of cPLA$_2$ GIVA. The generation of these cPLA$_2$ GIVA$^{-/-}$ mice and their initial characterization has been described previously (Uozumi et al., 1997). The role of iPLA$_2$ GVIA was assessed by treating C57BL/6 mice with daily intraperitoneal injections of a novel fluoroketone that is a selective inhibitor for iPLA$_2$ GVIA (FKGK11; 200 μl of a 2 mM solution). FKGK11 is a fluoroketone that is highly selective for iPLA$_2$ GVIA as it shows >95% inhibition of iPLA$_2$ at 0.091 mole fraction as compared to only 17% for cPLA$_2$. At this high concentration of substrate, values below 25% are not considered significant; the $X_i(50)$ value of FKGK11 for iPLA$_2 = 0.0073 \pm 0.00074$. $X_i(50)$ is the mole fraction of the inhibitor in the total substrate interface required to inhibit the enzyme by 50%, indicating that FKGK11 is a potent inhibitor of iPLA$_2$ (Baskakis et al., manuscript submitted). Mice in the control group were treated with the vehicle used to suspend the inhibitor, i.e. PBS containing 5% Tween 80. In addition, one group of cPLA$_2$ GIVA$^{-/-}$ mice were also given FKGK11 at the same dose to assess the combined effects of cPLA$_2$ GIVA and iPLA$_2$ GVIA.

Functional evaluation
Prior to lesion and at 7, 11, 14, 17 and 21 days following crush injury, mice were allowed to walk down a 40 cm long track after inking their hind paws. From the footprints, the sciatic functional index (SFI) was calculated using the formula developed by de Medinaceli and co-workers (de Medinaceli et al., 1982) and modified for mice by Insierra and co-workers (Inserra et al., 1998).

Recovery of pain sensitivity was tested on mice by pinching the most distal portion of the last three toes (third, fourth and fifth) of the injured hind limb with forceps. Digits 1 and 2 were not tested since they are partially innervated by the saphenous nerve. The first day after the lesion on which foot withdrawal was observed was recorded (Siconolfi and Seeds, 2001). All analyses were carried out blind.

Double immunofluorescence
Mice were deeply anesthetized as described above at 6 h, 2, 5, 7 and 21 days after the crush injury and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). A 5 mm length of the sciatic nerve distal to the site of crush was cryoprotected and longitudinal cryostat sections (10 μm) collected on permanent positive-charged surface slides. The crush site was identified using a 10-0 suture. Tissue sections were blocked in

0.1% Triton-X 100 and 1% normal goat serum and incubated overnight with polyclonal anti-cPLA$_2$ (Santa Cruz Biotechnology, 1:75) or anti-iPLA$_2$ (Cayman Chemicals, 1:500), combined with monoclonal antibodies specific for Schwann cells (mouse anti-S100β, Sigma, 1:200) or macrophages (rat anti-Mac-1, Serotec 1:200). This was followed by incubation with donkey anti-rabbit fluorescein-conjugated secondary antibody (Jackson Immuno-Research Laboratories, West Grove, PA, 1:400) combined with a donkey anti-mouse or anti-rat rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, 1:400). Tissue sections were examined using a Zeiss AxioSkop 2 FS Plus microscope (Carl Zeiss Canada, Toronto, Canada) equipped with flurorescence optics (filter set 9 and 15).

Assessment of axonal regeneration and skin re-innervation

Four days after the nerve crush, animals ($n = 3$ per group) were deeply anesthetized and transcardially perfused with 4% PFA as described above. A 5 mm length of the sciatic nerve distal to the site of crush injury was prepared for cryostat sectioning and serial cross sections (10 μm) taken from 3 mm distal to the crush were collected on positively charged glass slides. Tissue sections were blocked in 0.1% Triton-X 100 with 2% goat normal serum for 4 h and incubated overnight at 4°C with rabbit polyclonal antibodies against GAP-43 (1:500, Chemicon) and a monoclonal mouse anti-S100β (Sigma, 1:200) followed by a 1 h incubation at room temperature with goat anti-rabbit fluorescein-conjugated secondary antibody (1:500) combined with a donkey anti-mouse rhodamine-conjugated secondary antibody (1:400) (Jackson ImmunoResearch Laboratories). Axonal regeneration was assessed by counting the number of GAP-43 positive and S100 negative fibres at 3 mm distal to the crush site.

Twenty one days after the crush injury, animals ($n = 6$ per group) were perfused with 4% PFA, and plantar pads from the injured hind paw were harvested, post-fixed, cryoprotected and cut with a cryostat (25 μm). Tissue sections were immunostained as described above using rabbit polyclonal antibodies against PGP 9.5 (1:500, Chemicon, Temecula, CA), a marker for all types of nerve fibres, followed by a fluorescein-conjugated goat anti-rabbit secondary antibody.

Histological analysis

At 7 days ($n = 3$ per group) and 21 days ($n = 6$ per group) after the crush injury, mice were deeply anesthetized and perfused with 0.1 M phosphate buffer followed by 3% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M phosphate buffer. The distal segment of the sciatic nerve was cut into 1 mm segments, post-fixed in 2% osmium tetroxide for 2 h, and embedded in Epon. Cross sections (1 μm) of the nerve were cut and stained with 1% toluidine blue for light microscopy.

Images of the whole sciatic nerve sampled at 3 mm distal to the site of crush were captured at ×10 with a Retiga 1300C digital camera (QImaging Corp., Burnaby, British Columbia) using a Zeiss AxioSkop II (Carl Zeiss Canada Ltd., Toronto) light microscope to assess the area of the nerve. In addition, six sets of images chosen by random sampling of squares representing at least 40% of the nerve cross-sectional area were also acquired at ×100. These images were used to calculate the numbers of myelinated degenerating fibres and macrophages. Macrophages were identified by their 'foamy' morphology in 1 μm thick Epon

embedded cross sections of the nerve stained with toluidine blue. The 'foamy' morphology which is due to the presence of end-products of myelin/lipid degradation, is widely used to identify phagocytic macrophages (Boven *et al.*, 2006). Degenerating fibres that were not phagocytosed were distinguished into two types: ones with disrupted myelin sheaths; ones with more compact myelin sheaths. The latter were identified as fibres in the process of degeneration because of changes in the axoplasm or presence of additional whorls of compact myelin. Degenerating fiber counts, are expressed as a percentage of the total number of fibres in the uninjured sciatic nerve. All analyses were carried out blind.

Electron microscopy

Ultrathin cross sections of Epon embedded sciatic nerves ($n = 3$ for each group) at a distance of 4 mm distal to the crush site were cut at 7 days after the lesion. Sections were stained with lead citrate and viewed with a Philips CM 10 electron microscope. Large single unmyelinated axons ensheathed by Schwann cells were counted in each nerve in a total area of $2.7 \times 10^4 \, \mu m^2$. Data are presented as the mean ± SEM. Statistical analysis was performed as described below.

Quantitative real-time PCR

A 10 mm length of nerve distal to the lesion was harvested from uninjured mice and at 1 day after crush injury and RNA extracted using the RNeasy Lipid Tissue kit (Qiagen, Mississauga, Ontario, Canada). Nerves from eight mice were pooled for each group. A reverse transcription (RT) reaction was then carried out using Omniscript® RT kit (Qiagen, Mississauga, ON) according to the manufacturer's protocol. One μl of the RT product was added to 24 μl of Brilliant SYBR Green quantitative PCR Master Mix (Stratagene), and QRT-PCR was done to analyse the expression of IL-1β and MCP-1 (MX4000 apparatus, Stratagene). The primers 5′-TCAGGCAGGCAGTATCACT-3′ (sense) and 5′-CACGGGAAA GACACAGGTAGCT-3′ (antisense); and 5′-GAGAGCTACAAG AGGATCACCA-3′ (sense) and 5′-GTATGTCTGGACCCATTCCT TC-3′ (antisense) were used to amplify IL-1β and MCP-1 cDNA, respectively. Peptidylprolyl isomerase A (PPIA) was used as a housekeeping gene using the following primers: 5′-AGCATAC AGGTCCTGGCATC-3′ (sense) and 5′-TTCACCTTCCCAAAGA CCAC-3′ (antisense). The amount of cDNA was calculated based on the threshold cycle (C_T) value, and was standardized by the amount of house-keeping gene using the $2^{-\Delta \Delta C_T}$ method (Livak and Schmittgen, 2001).

Statistical analyses

Data are shown as mean ± SEM. The histological data was analysed by the Student's *t*-test. Statistical analyses of functional recovery were performed using two-way repeated-measures ANOVA with post-hoc Tukey's test for multiple comparisons. Differences were considered significant at $P < 0.05$.

Results

Expression of cPLA$_2$ GIVA and iPLA$_2$ GVIA in Wallerian degeneration

We first assessed using immunofluorescence the localization of cPLA$_2$ GIVA and iPLA$_2$ GVIA in Wallerian degeneration after sciatic nerve crush injury. No detectable
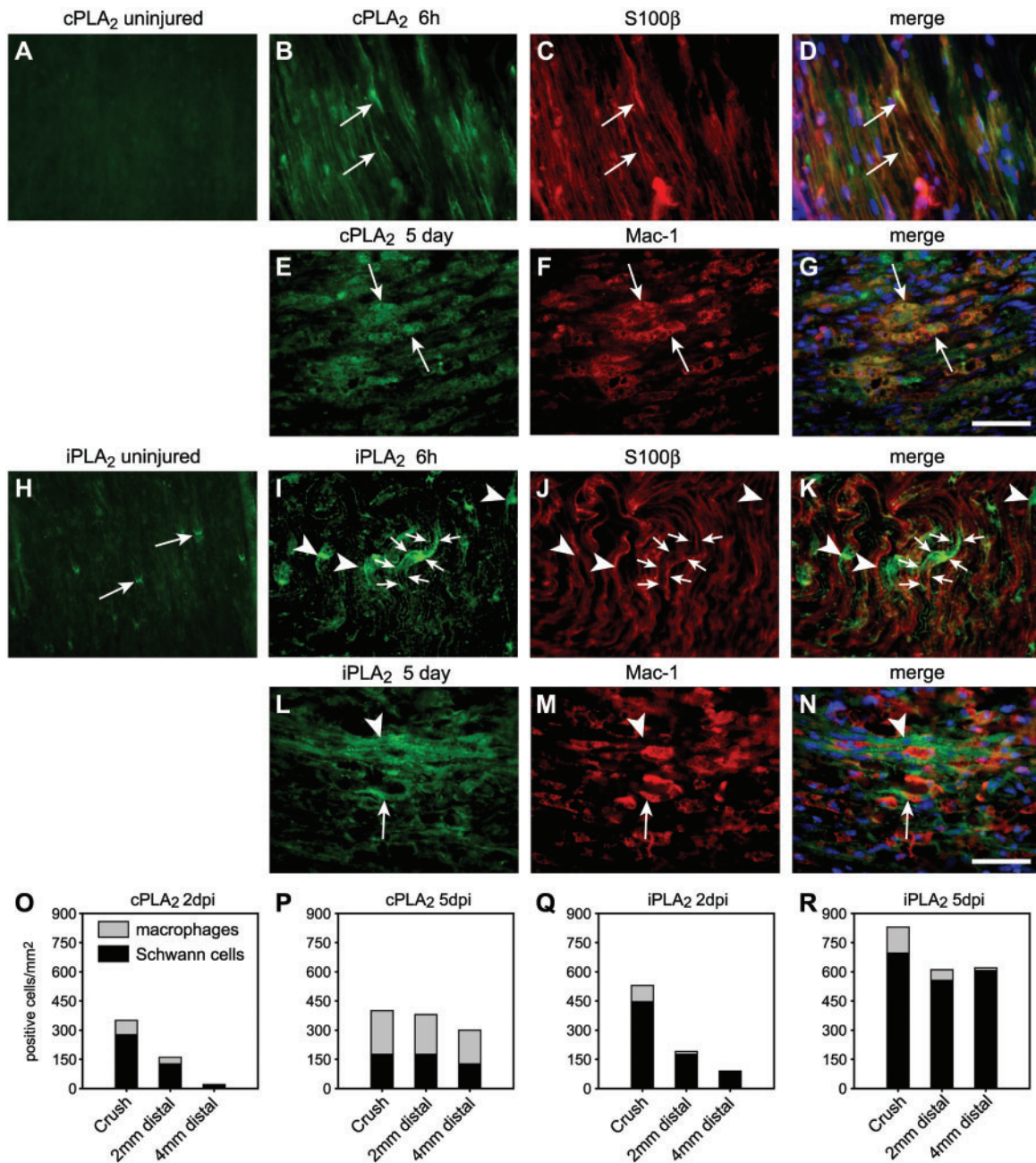
Fig. I Expression of cPLA$_2$ GIVA and iPLA$_2$ GVIA in the uninjured and injured mouse sciatic nerve. (**A**) cPLA$_2$ GIVA is not expressed in detectable levels in the uninjured mouse sciatic nerve. (**B–D**) As early as 6 h after crush injury cPLA$_2$ GIVA is expressed near the site of lesion in S100β$^+$ Schwann cells (arrows). (**E–G**) After 5 days, cPLA$_2$ GIVA expression is robust mainly in Mac-1$^+$ phagocytic macrophages (arrows). (**H**) In uninjured sciatic nerve, iPLA$_2$ GVIA is expressed at the paranodal loop regions of the myelin sheath (arrows). (**I–K**) At 6 h after crush injury, iPLA$_2$ immunoreactivity spreads from the paranodal regions along the length of the myelin sheath (arrows). Tissue section double-labelled with S100β. (**L–N**) At 5 days after injury, iPLA$_2$ immunoreactivity was found both in Mac-1$^+$ macrophages (arrow) and Mac-1 negative elongated cells. The latter are Schwann cells based on labelling with S100β (data not shown). Scale Bars = 50 μm. (**O–R**) Quantification of the number of cPLA2$^+$ (**O**) and iPLA$_2$$^+$ (**Q**) Schwann cells (black bars) and macrophages (grey bars) at 2 days post injury (dpi) show a greater number of these cells at the site of crush and decrease distally along the nerve. At 5 dpi, 60% of the cPLA$_2$ + cells are macrophages (**P**), while iPLA$_2$ is expressed mainly by Schwann cells (**R**). Data is the mean from three experiments. See supplemental table.

immunostaining for cPLA$_2$ GIVA was found in the sciatic nerve of uninjured mice (Fig. 1A). Six hours after injury, cPLA$_2$ GIVA was exclusively observed in the site of the crush and was co-localized with S100β staining, indicating that the early expression of cPLA$_2$ GIVA is in Schwann cells

(Fig. 1B–D). Expression of cPLA$_2$ GIVA progressively extended caudal to the crush starting at Day 2 after injury and occupied most of nerve length by Day 5 (Fig. 1O and P). Double immunofluorescence revealed that at 2 days after injury cPLA$_2$ GIVA was expressed mainly in Schwann

cells, while at 5 days post lesion ∼60% of the cPLA$_2$$^+$ cells were macrophages (Fig. 1E–G, O and P; supplemental table). At 21 days post-injury, immunoreactivity for cPLA$_2$ was back to normal, i.e. almost undetectable (Supplemental Fig. 1A).

Immunoreactivity for iPLA$_2$ GVIA in the uninjured sciatic nerve was detectable in the paranodal loop regions of the myelin sheath (Fig. 1H). Six hours after the injury, iPLA$_2$ GVIA immunoreactivity at the lesion site was more intense and spread beyond the paranodal region along the myelin sheath (Fig. 1I–K). At 2 days after injury, iPLA$_2$ immunoreactivity extended distal to the site of crush, occupying most of entire length by Day 5 post-injury (Fig. 1Q and R). iPLA$_2$ was expressed mainly in Schwann cells in the injured nerve, although some macrophages were also iPLA$_2$$^+$ (Fig. 1I–N, Q and R; supplemental table). By Day 21 after crush, iPLA$_2$ GVIA immunoreactivity was again restricted to the paranodal regions of the myelin sheath (Supplemental Fig. 1B).

cPLA$_2$ GIVA and iPLA$_2$ GVIA are involved in Wallerian degeneration

To obtain direct evidence whether cPLA$_2$ GIVA and iPLA$_2$ GVIA play a role in Wallerian degeneration, we crushed the right sciatic nerve in cPLA$_2$ GIVA$^{-/-}$ mice and C57BL/6 mice receiving a daily injection of FKGK11, a novel selective iPLA$_2$ inhibitor ($n = 3$ per group). Seven days post-injury, sciatic nerves from wild-type control animals showed advanced signs of breakdown of compact myelin and myelin phagocytosis (Fig. 2A and 5A). In wild-type-injured nerves, toluidine-blue stained sections 3 mm caudal to the crush displayed numerous phagocytic macrophages, which appear to have phagocytosed most of the degenerating myelin (∼90% of the total fibres) (Fig. 2A, 2E–G). The presence of degenerating fibres with compact myelin sheaths was scarce (Fig. 2G). Sciatic nerves from mice lacking cPLA$_2$ GIVA or treated with iPLA$_2$ inhibitor (FKGK11) showed delayed signs of myelin breakdown (Fig. 2B and C) and contained fewer phagocytic macrophages (40% reduction in cPLA$_2$ GIVA$^{-/-}$ mice, and 30% reduction in mice treated with the FKGK11; Fig. 2E) and greater numbers of unphagocytosed nerve fibres with disrupted myelin sheaths that are still in the process of degeneration (100% increase in cPLA$_2$ GIVA$^{-/-}$, and 50% in mice treated with FKGK11; Fig. 2F). In addition, the number of unphagocytosed fibres that still had compact myelin sheaths was about 7.5 and 13-fold-higher in mice lacking cPLA$_2$ GIVA and in wild-type mice treated with the FKGK11, respectively (Fig. 2G). The fibres with the compact myelin sheaths in these mice are not likely to be newly regenerated and remyelinated axons because of their large diameter and the thickness of their myelin sheaths. Furthermore, the axoplasm shows signs of degeneration and there are often additional myelin whorls—evidence that these fibres are in the process of degeneration (Fig. 5B

and D). Regenerating axons at this early stage (7 days) after injury are not yet myelinated (see section below). These data suggest that although both intracellular forms of PLA$_2$ are involved in Wallerian degeneration, iPLA$_2$ GVIA is likely to play a greater role in the early stages of myelin breakdown, while cPLA$_2$ GIVA may be more involved in the later stages of myelin clearance by macrophages.

We then assessed whether the lack of activity of both cPLA$_2$ GIVA and iPLA$_2$ GVIA leads to a greater delay in Wallerian degeneration. For these experiments, cPLA$_2$ GIVA$^{-/-}$ mice were given daily injections of FKGK11. At 7 days post injury, sciatic nerve sections from animals lacking activity of both PLA$_2$s showed a reduction in the number of macrophages that was similar to that of cPLA$_2$ GIVA null mice (Fig. 2). They also had similar numbers of degenerating fibres with disrupted myelin sheaths as cPLA$_2$ GIVA$^{-/-}$ mice (Fig. 2F and G) while the number of fibres with compact myelin sheaths were similar to that of mice treated with FKGK11 (Fig. 2F and G). The presence of degenerating nerve fibres with compact myelin sheaths was also evident by electron microscopy (Fig. 5D). These results suggest that iPLA$_2$ may contribute more to the early phase of myelin breakdown, while cPLA$_2$ may contribute more to myelin phagocytosis by macrophages.
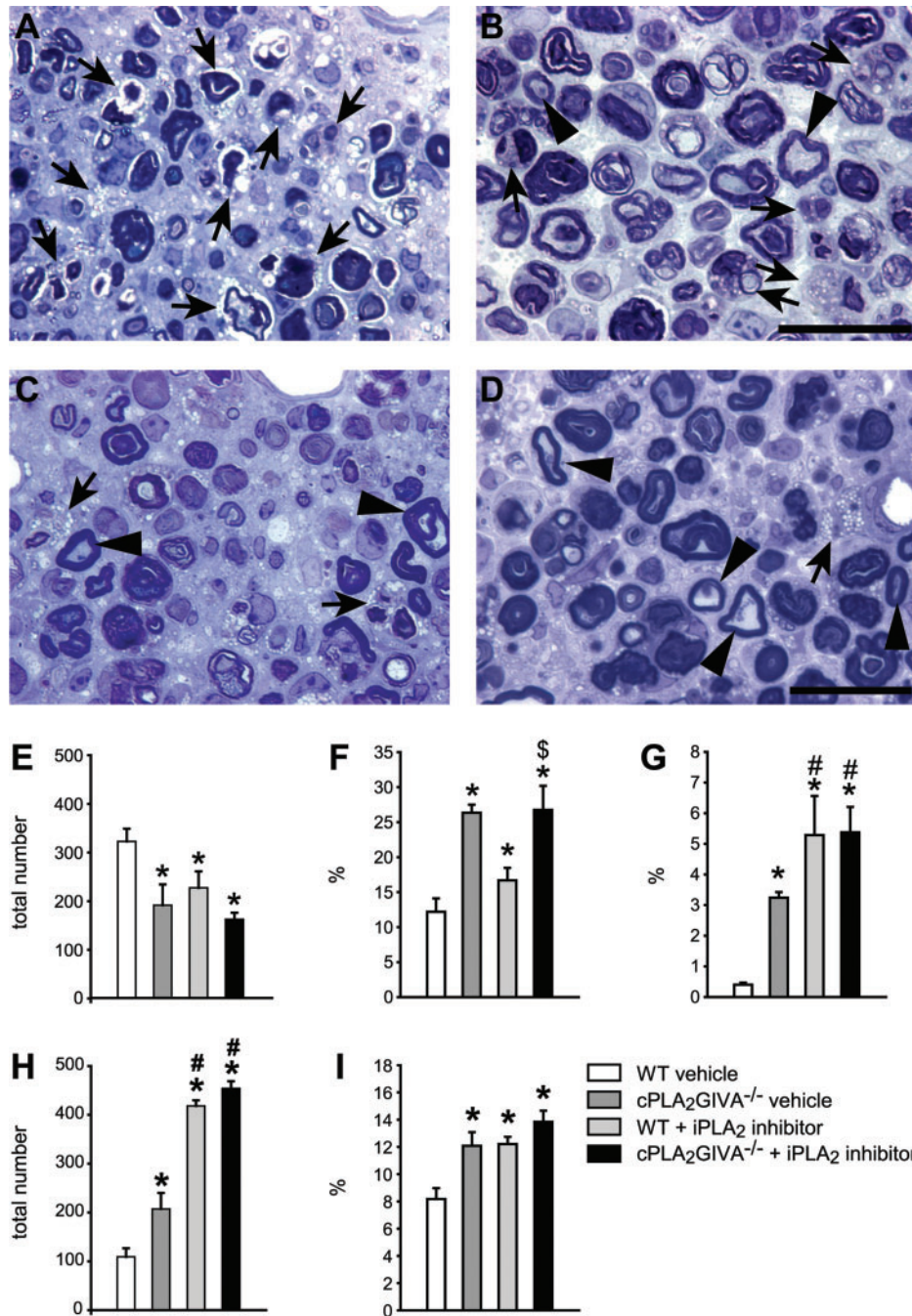
We then assessed changes in the sciatic nerves at 21 days after crush ($n = 6$ per group), when almost all of the myelin debris has been removed and axon regeneration has occurred in the wild-type control mice. At this time point, and in contrast to 7 days post injury, sciatic nerves from cPLA$_2$ GIVA$^{-/-}$ mice and wild-type littermates treated with FKGK11 showed a significant increase in the number of macrophages and degenerating fibres with disrupted myelin sheaths (Fig. 2H and I), further indicating that Wallerian degeneration was delayed with the lack of cPLA$_2$ GIVA or iPLA$_2$ GVIA activity. Most of the phagocytic macrophages have been cleared from the nerve at 21 days in wild-type mice (Fig. 2H). Interestingly, mice treated with FKGK11 had 2-fold higher number of phagocytic macrophages than cPLA$_2$ GIVA$^{-/-}$ mice, suggesting that iPLA$_2$ GVIA could also contribute to the efflux of macrophages from the nerve at later stages of Wallerian degeneration. Similar changes were also observed in mice lacking both PLA$_2$ activities (Fig. 2H).

These data provide direct evidence that cPLA$_2$ GIVA and iPLA$_2$ GVIA play an important role in myelin breakdown and phagocytosis after peripheral nerve injury, and the lack of their activity results in impaired Wallerian degeneration.
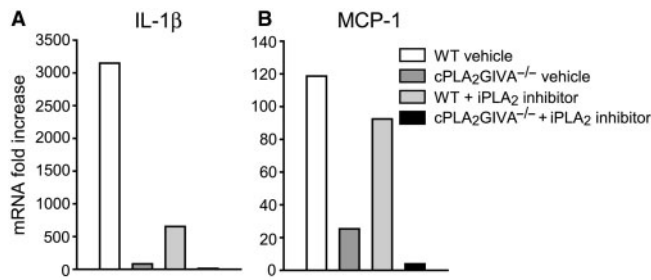
Altered expression of pro-inflammatory mediators in the absence of cPLA$_2$ GIVA and/or iPLA$_2$ GVIA activity

Chemokines and cytokines are important regulators of the immune response. Among them, IL-1β and MCP-1 play a crucial role in the recruitment and activation of macrophages in injured peripheral nerve (Shamash *et al.*, 2002;

Fig. 2 Effect of cPLA₂ GIVA and iPLA₂ GVIA on Wallerian degeneration in the sciatic nerve. (**A**) Epon embedded toluidine blue-stained cross section of the lesioned sciatic nerve of a wild-type mouse 7 days after nerve crush injury shows marked disruption of myelin and phagocytosis by macrophages (arrows). In contrast, the sciatic nerve of a cPLA₂ GIVA$^{-/-}$ mouse (**B**), wild-type mouse treated with the iPLA₂ inhibitor (FKGKII) (**C**), or cPLA₂ GIVA$^{-/-}$ mouse treated with FKGKII (**D**), show large numbers of well preserved intact myelin profiles (arrowheads), and fewer phagocytic macrophages (arrows). Graphs show the quantification of the total number of phagocytic macrophages (**E**), degenerating fibres with disrupted myelin sheaths (**F**) and fibres with intact myelin sheaths (**G**) in cross-sections of lesioned sciatic nerves from wild-type vehicle-treated mice, cPLA₂ GIVA$^{-/-}$, wild-type mice treated with the iPLA₂ inhibitor, and cPLA₂ GIVA$^{-/-}$ mice treated with the iPLA₂ inhibitor at 7 days after crush injury. Values in F and G represent the number of degenerating fibres with disrupted myelin sheath (F) and fibres with compact myelin sheaths (G), shown as a percentage of the total number of fibres in the uninjured nerve. Note the marked reduction in the number of macrophages in the cPLA₂ GIVA null mice and iPLA₂ inhibitor treated mice as compared to the wild-type mice (E), and the marked slowing of myelin breakdown and phagocytosis (**F** and **G**). The quantification at 21 days after crush injury of the total number of phagocytic macrophages (**H**) and degenerating fibres with disrupted myelin sheath (I) shown as a percentage of the total number of myelinated fibres in the uninjured nerve. Note the markedly increased persistence of phagocytic macrophages (H) in mice treated with the iPLA₂ inhibitor as compared to the cPLA₂ null mice and injured wild-type controls.
Bars = 25 μm; *$P < 0.05$ for experimental groups versus wild-type (vehicle) controls; $ = $P < 0.05$ for indicated groups versus FKGKII-treated mice; # = $P < 0.05$ for indicated groups versus cPLA₂$^{-/-}$ mice.
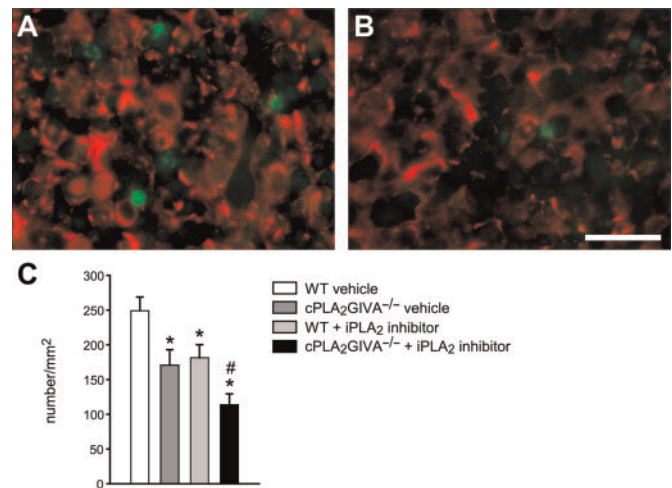
Fig. 3 Q-PCR analyses of IL-1β and MCP-1 mRNA at 1 day after sciatic nerve crush injury. Values are expressed as fold increase over uninjured wild-type nerves. Note the marked increase in expression of IL-1β and MCP-1 after injury in wild-type vehicle treated mice. In contrast, the expression of IL-1β and in MCP-1 mRNA in mice lacking cPLA₂ GIVA is markedly less than in injured wild-type nerve. In wild-type mice treated with the iPLA₂ inhibitor, the expression of IL-1β mRNA is also lower but there is only a slight change in MCP-1. The expression of IL-1β and MCP-1 mRNA is more drastically decreased in cPLA₂ null mice treated with the iPLA₂ inhibitor as compared to injured wild-type vehicle-treated mice. PPIA is used as a housekeeping gene.

Perrin *et al.*, 2005). We therefore assessed, whether the decreased macrophage influx observed in cPLA₂ GIVA$^{-/-}$ mice and in mice treated with the iPLA₂ GVIA inhibitor was related to a reduction in the expression of these inflammatory mediators. Q-PCR performed on samples harvested at 1 day after crush (a pool of eight nerves per group) revealed a marked upregulation in IL-1β (3147-fold) and MCP-1 (118-fold) mRNA expression in the crushed sciatic nerve of WT-vehicle treated mice. In mice lacking cPLA₂ GIVA, the mRNA expression of IL-1β and MCP-1 was markedly less that in the injured vehicle treated wild-type (Fig. 3A and B), while wild-type mice treated with the iPLA₂ inhibitor also showed lower levels of IL-1β mRNA but little change in MCP-1 (Fig. 3A and B). The mRNA levels of IL-1β and MCP-1 were even lower in mice lacking both cPLA₂ GIVA and iPLA₂ activities (Fig. 3). The expression of these molecules was not completely blocked, as their levels were still 9-fold higher than in uninjured nerves, suggesting that other factors also contribute to this effect.

Axonal regeneration and functional recovery after sciatic nerve injury is impaired in the lack of cPLA₂ GIVA and/or iPLA₂ GVIA activity

We next carried out experiments to assess whether the reduction in myelin clearance by macrophages in the absence of cPLA₂ GIVA or iPLA₂ GVIA activity influences axon regeneration since peripheral nerve myelin contains molecules that inhibit axonal growth. We first quantified the number of GAP-43 positive axons that were S100β negative (Fig. 4A–C) in sciatic nerve cross-sections at a distance of 3 mm distal to the site of crush, 4 days after injury. As S100β-positive non-myelinating Schwann cells
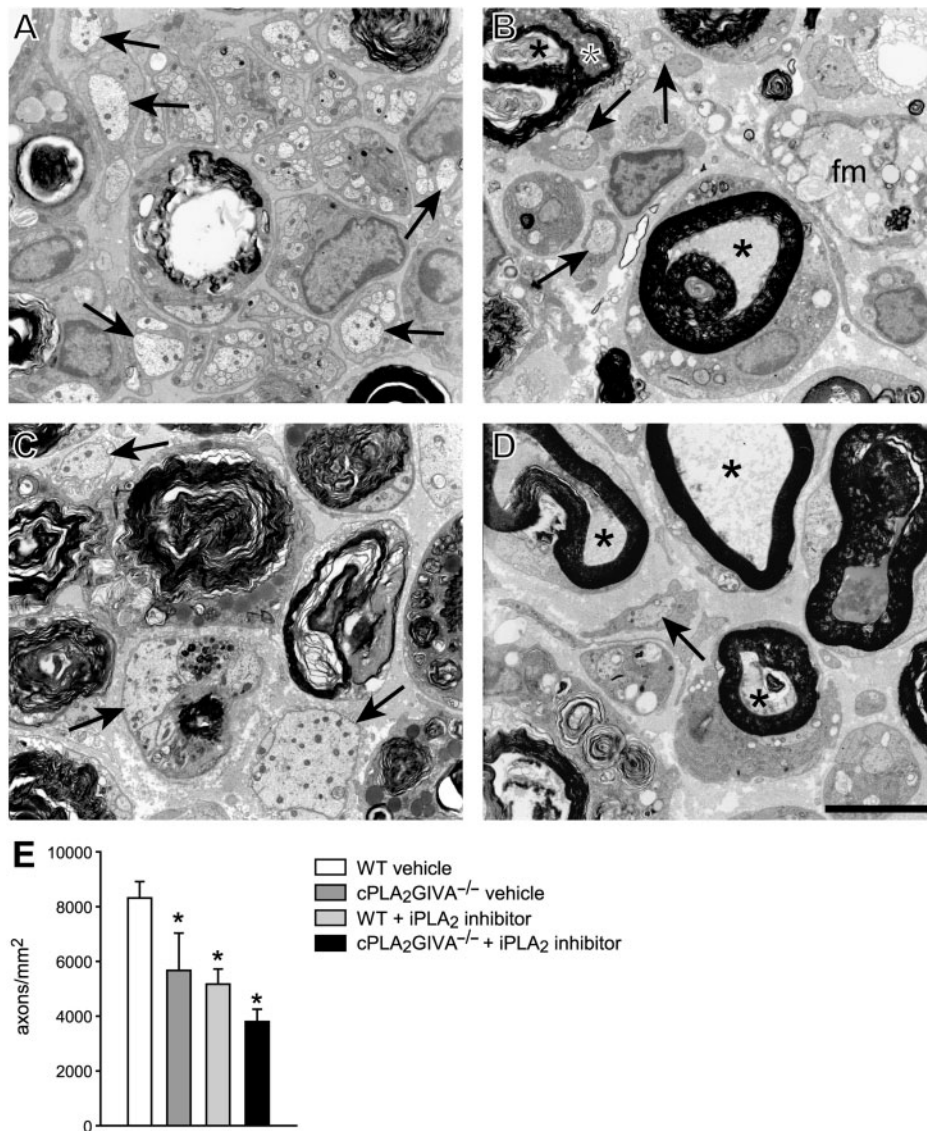

Fig. 4 Axon regeneration assessed 3 mm distal to the crush site at 4 days after sciatic nerve crush injury. Micrographs of cross-sections of sciatic nerve from a WT vehicle-treated mouse (**A**) and a cPLA₂ GVIA$^{-/-}$ mouse treated with iPLA₂ inhibitor (**B**) double-labelled with GAP-43 (green) and SI00β (red). Regenerating axons are identified as profiles that are labelled with GAP-43 but not SI00β. (**C**) Graph showing the quantification of GAP-43$^+$ regenerating axons. Note that, there is a significantly lower number of regenerating axons in cPLA₂ GIVA$^{-/-}$ mice and wild-type mice treated with the iPLA₂ inhibitor as compared to wild-type injured controls (*P < 0.05). There is also a further reduction in the number of regenerating axons in cPLA₂ GIVA$^{-/-}$ mice treated with the iPLA₂ inhibitor; $^{#}P < 0.05$ versus cPLA₂ null; and WT+iPLA₂ inhibitor). Bar = 20 μm.

may also express GAP-43 (Curtis *et al.*, 1992), we only quantified GAP-43 labelled rounded profiles that were S100β-negative to ensure we were counting regenerating axons and not Schwann cells (Fig. 4A). Lack of the cPLA₂ GIVA or iPLA₂ GVIA activity resulted in ∼30% reduction in the number of regenerating axons (Fig. 4C). In cPLA₂$^{-/-}$ mice treated with the iPLA₂ inhibitor (i.e. lacking both PLA₂s), the number of regenerating fibres was reduced even further to 55% of control values (Fig. 4C). To further confirm these results, we quantified the number of regenerated axons by electron microscopy in cross-sections of the sciatic nerve 4 mm distal to the crush site, at 7 days after injury. At this time point, many regenerated axons were present in the wild-type-vehicle treated injured nerve but these newly regenerated single large diameter axons were not yet myelinated. Mice lacking cPLA₂ GIVA or treated with the iPLA₂ inhibitor had significantly fewer regenerated axons (∼40%) as compared to the wild-type-vehicle treated mice (Fig. 5B, C, E). In addition, nerves from cPLA₂ GIVA null mice treated with the iPLA₂ inhibitor showed an even greater reduction in the number of single, large diameter regenerated axons (∼60%) (Fig. 5D and E). These results provide direct evidence that the lack of intracellular forms of PLA₂ lead to delayed axonal regeneration after sciatic nerve injury.

We further evaluated the rate of functional recovery after sciatic nerve crush injury in mice lacking cPLA₂ GIVA or
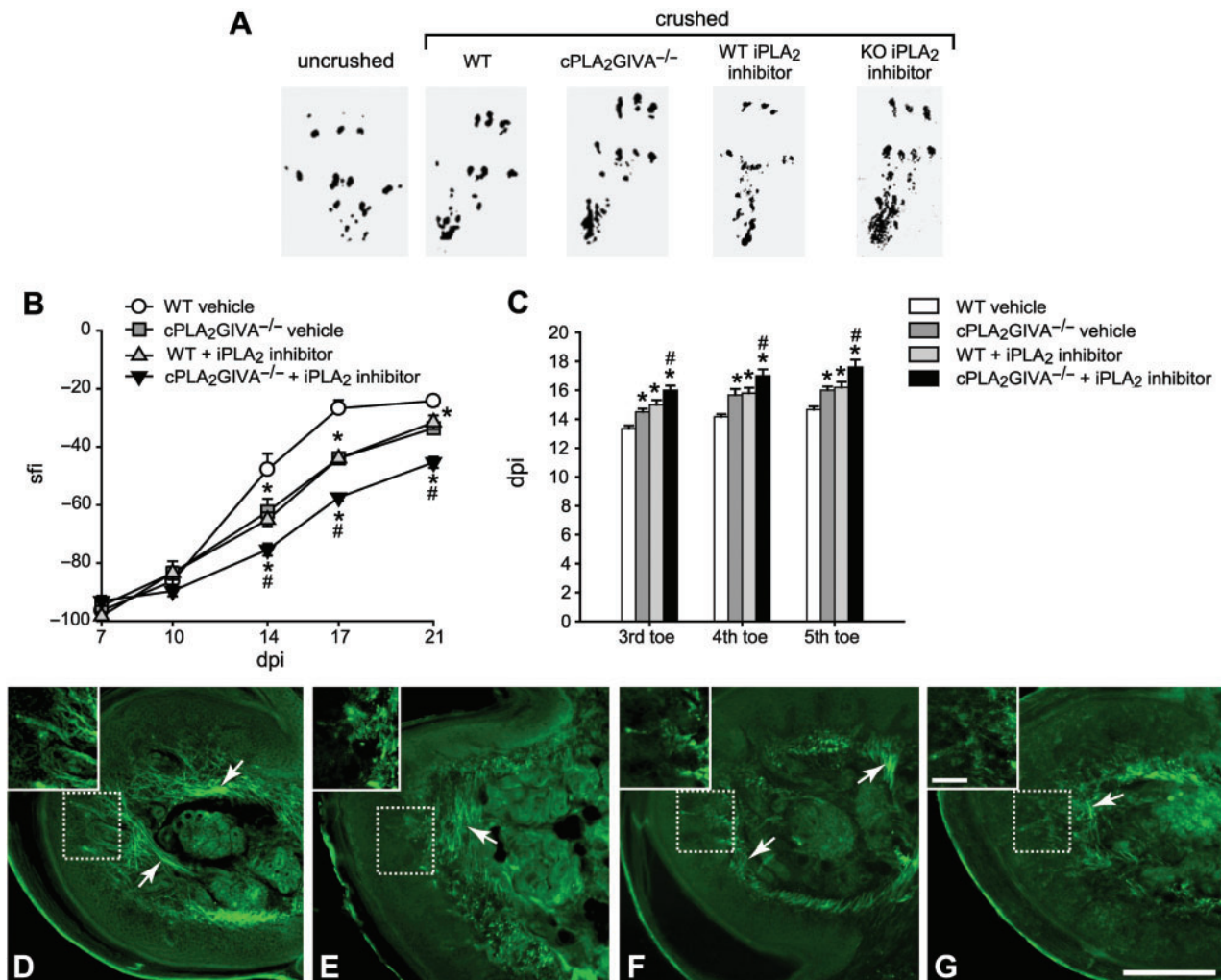
Fig. 5 Electron micrographs of nerves 4 mm distal to the crush site at 7 days after injury from wild-type (WT) vehicle-treated mouse (**A**), WT iPLA$_2$ inhibitor treated (**B**), cPLA$_2^{-/-}$ mouse (**C**), and cPLA$_2^{-/-}$ mouse treated with the cPLA$_2$ inhibitor (**D**). Note the single large diameter, regenerated axons that are not yet myelinated (arrows), Note also the presence of degenerating fibres with compact myelin sheaths (*) in nerves of WT mice treated with the iPLA$_2$ inhibitor (**B**) and cPLA$_2^{-/-}$ mice treated with the iPLA$_2$ inhibitor (**D**). A 'foamy' macrophage (fm) is seen in B. (**E**) Graph showing quantification of these regenerating axons ($n = 3$); $^*P < 0.05$ as compared to WT injured controls. Scale Bar = 5 µm.

iPLA$_2$ GVIA activity. The walking track method in which foot-prints are analysed was used to assess motor recovery. After sciatic nerve crush, the SFI calculated from foot-prints by measuring the distance between the first and the fifth toes and the length of the footprint (Fig. 6A), drops immediately to a negative value of about −100, indicating a marked disability in the injured paw (Fig. 6B). This deficit recovers towards the normal value of 0 when axons regenerate and appropriately reinnervate their targets (Fig. 6B). Wild-type control littermates underwent a fast recovery in the SFI from 10 to 17 days post injury, showing toe spreading and plantar length close to that of the

uninjured paw (Fig. 6A and B). Mice lacking cPLA$_2$ GIVA or iPLA$_2$ GVIA activity showed significantly slower recovery in SFI compared to control injured mice (Fig. 6A and B). This deficit in functional recovery was even greater in mice lacking both cPLA$_2$ GIVA and iPLA$_2$ GVIA activity (Fig. 6A and B).

We also examined the recovery of sensory function by means of the toe pinch reflex. After sciatic nerve injury, the pinch reflex response of the third, fourth and fifth, toe was abolished in all animals. Recovery of this response in the fifth toe was observed by 14 days after injury in wild-type mice, and delayed by 2 days in cPLA$_2$ GIVA$^{-/-}$ mice and

Fig. 6 Effect of cPLA$_2$ GIVA and iPLA$_2$ GVIA on functional recovery. (**A**) Representative footprints obtained from uninjured wild-type mice, and 21 days after sciatic nerve crush injury in wild-type mice, cPLA$_2$ GIVA$^{-/-}$ mice, wild-type mice treated with the iPLA$_2$ inhibitor (FKGK11), and cPLA$_2$ GIVA$^{-/-}$ mice treated with FKGK11. At 21 days post-injury, footprints from the injured paw of wild-type mice (WT) display a certain degree of recovery in toe spreading and length of the foot-print due to axonal regeneration and re-innervation. Note that this recovery is impaired in cPLA$_2$ GIVA$^{-/-}$ mice, and wild-type mice treated with the iPLA$_2$ inhibitor (WT FKGK11), and cPLA$_2$ GIVA$^{-/-}$ mice treated with the iPLA$_2$ inhibitor (KO FKGK11) as compared to wild-type mice (WT). Foot-print analysis shows narrower toe spreading and longer footprint length in cPLA$_2$$^{-/-}$ mice and mice treated with the iPLA$_2$ inhibitor than the wild-type controls. (**B**) Graph showing the recovery of motor function (footprints) after sciatic nerve crush determined by the SFI (described in the Methods section). Delayed recovery of motor function (SFI index) is evident in mice lacking the activity of either cPLA$_2$ GIVA or iPLA$_2$ GVIA or both PLA$_2$s as compared to wild-type controls. (**C**) Histogram showing the days post-injury after sciatic nerve crush at which the toe pinch reflex was first elicited for the third, fourth and fifth, toes. Recovery of the toe pinch withdrawal reflex is delayed by about 2 days in mice lacking cPLA$_2$ GIVA or wild-type mice treated with the iPLA$_2$ inhibitor. The combined lack of both PLA$_2$ activities results in an even further delay of 3 days. (**D**–**G**) Re-innervation of the plantar foot-pads at 21 days after sciatic nerve crush in wild-type (**D**) cPLA$_2$ GIVA$^{-/-}$ (**E**), wild-type treated with the iPLA$_2$ inhibitor (**F**), and cPLA$_2$ GIVA$^{-/-}$ treated with the iPLA$_2$ inhibitor (**G**). Note that the PGP 9.5 immunoreactive fibres (arrows) are seen around the sweat glands and also close to the sub-epidermal nerve plexus in wild-type animals treated with vehicle (D), whereas fewer labeled fibres are seen in the other groups. Inserts show higher magnification images of the areas outlined by the hatched squares. Scale Bar = 200 μm; scale bar in insert = 50 μm. *$P < 0.05$ as compared to WT injured controls; $^\#P < 0.05$ as compared to cPLA$_2$ null; and WT+iPLA$_2$ inhibitor.

animals treated with the iPLA$_2$ inhibitor (Fig. 6C). Lack of activity of both PLA$_2$s led to a further delay (3 days) in recovery in pinch reflex response (Fig. 6C).

Finally, we studied the regeneration and innervation of PGP 9.5 positive fibres within the sweat glands (autonomic fibres) and skin of the hind paw (sensory fibres). At 21 days after crush injury, all wild-type control mice showed many PGP 9.5 immunoreactive axonal profiles re-innervating the sweat glands and skin of the injured hind paw (Fig. 6D). In contrast, very few PGP 9.5 immunoreactive fibres were found innervating the sweat glands and skin of cPLA$_2$ GIVA$^{-/-}$ mice (Fig. 6E), mice treated with the iPLA$_2$ inhibitor (Fig. 6F), or cPLA$_2$ GIVA$^{-/-}$ mice treated with the iPLA$_2$ inhibitor (Fig. 6G).

These results show that, axon regeneration and re-innervation is delayed in the absence of lack of cPLA$_2$ GIVA and iPLA$_2$ GVIA.

Discussion

The results of this study provide direct evidence that cPLA$_2$ GIVA and iPLA$_2$ GVIA play a significant role in Wallerian degeneration in peripheral nerve injury. This is based on studies done on the cPLA$_2$ GIVA deficient mice and wild-type mice treated with a selective inhibitor for iPLA$_2$ GVIA in which myelin breakdown, macrophage recruitment and myelin phagocytosis are severely impaired. We also show that these changes are accompanied with a deficit in axon regeneration, target re-innervation and functional recovery.

Schwann cells are thought to play a key role in the initiation of myelin degradation after peripheral nerve injury because this process is initiated within the first few hours after injury before the influx of macrophages. We show that Schwann cells express iPLA$_2$ GVIA and cPLA$_2$ GIVA within 6 h after sciatic nerve injury. PLA$_2$s hydrolyze membrane phospholipids to release fatty acids, such as AA, and a lysophospholipid, such as LPC. The latter is a potent demyelinating agent that can initiate myelin breakdown. Myelin is especially susceptible to LPC because, unlike other membranes, it does not possess or is not readily accessible to reacylating enzymes, which limit the damage (Gregson and Hall, 1973). The first signs of myelin degeneration after nerve injury are found at the paranodes and Schmidt–Lantermann incisures, which contain Schwann cell cytoplasm (Williams and Hall, 1971) and are also the first affected after intraneural injection of LPC or *Crotalus* venom PLA$_2$ (Hall and Gregson, 1971; Gregson and Hall, 1973; Hall, 1989). Therefore, the synthesis of LPC by PLA$_2$s at the paranodes and Schmidt–Lantermann incisures may trigger the onset of myelin breakdown. This is in keeping with our current findings that iPLA$_2$ immunoreactivity spreads from the paranodal regions along the myelin sheath after injury. Interestingly, when iPLA$_2$ GVIA activity was blocked, there was a 13-fold increase in the number of degenerating nerve fibres with intact or compact myelin sheaths as compared to vehicle treated mice, suggesting that iPLA$_2$ GVIA plays a role in the early stages of myelin breakdown. Interestingly, cPLA$_2$ GIVA is also expressed in Schwann cells, especially during the first few hours after injury, although its expression is more robust in macrophages at 5 and 7 days after injury. Mice lacking cPLA$_2$ GIVA, however, showed a 7.5-fold-increase in the numbers of degenerating fibres with compact myelin as compared to wild-type littermates. This suggests that cPLA$_2$ GIVA also plays a role in myelin breakdown, although to a lesser extent than iPLA$_2$ GVIA.

PLA$_2$s expressed in Schwann cells may also trigger the influx of macrophages into the injured tissue. Macrophages participate in Wallerian degeneration and play the main role in the phagocytosis of myelin and axonal debris.

We previously reported that AACOCF$_3$, a compound that blocks all members of the intracellular PLA$_2$s, including cPLA$_2$ GIVA and iPLA$_2$ GVIA, reduced ∼60% of the macrophages recruited into the transected sciatic nerve (De *et al.*, 2003). In the present study, a similar reduction in the number of macrophages was observed in cPLA$_2$ GIVA$^{-/-}$ mice treated with the iPLA$_2$ inhibitor. However, when we dissected out the contribution of each of these intracellular PLA$_2$s, we found that cPLA$_2$ GIVA appears to have a greater role in macrophage recruitment, since the number of macrophages was reduced by 40% in cPLA$_2$ GIVA$^{-/-}$ mice, as compared to 30% reduction in animals treated with the iPLA$_2$ inhibitor.

Recruitment of macrophages into the injured distal nerve segment is mediated in part by chemokines and cytokines, which are expressed by Schwann cells as early as 24 h after injury (Toews *et al.*, 1998; Shamash *et al.*, 2002; Perrin *et al.*, 2005). We found, that the expression of IL-1β and MCP-1 mRNA in mice lacking cPLA$_2$ GIVA was markedly less than in injured wild-type nerve. In wild-type mice treated with the iPLA$_2$ inhibitor there was also lower level of IL-1β mRNA but only a slight change in MCP-1. However, the expression of IL-1β and MCP-1 mRNA was more drastically decreased in cPLA$_2$ null mice treated with the iPLA$_2$ inhibitor as compared to injured wild-type vehicle-treated mice. The greater reduction in the expression of MCP-1 in cPLA$_2$ GIVA null mice as compared to mice treated with the iPLA$_2$ inhibitor correlates with the greater reduction in the number of phagocytic macrophages. We previously showed that LPC injected directly into the mouse spinal cord induces the expression of a number of pro-inflammatory chemokines and cytokines including IL-1β and MCP-1, and that neutralizing IL-1β or MCP-1 with blocking antibodies reduced LPC-induced macrophage influx and activation (Ousman and David, 2000, 2001). Furthermore, neutralizing IL-1β and MCP-1 with blocking antibodies also reduced macrophage recruitment, activation and myelin clearance in the injured mouse sciatic nerve (Perrin *et al.*, 2005). As well, AA produced by PLA$_2$s is metabolized by cyclooxygenase enzymes (COX-1 and COX-2) to prostaglandins, which are potent mediators of inflammation that can induce the recruitment of macrophages by promoting vasodilatation and expression of cytokines, such as IL-1β (Rocha *et al.*, 2003). Taken together, these findings suggest that PLA$_2$ enzymes expressed by Schwann cells may induce the recruitment and activation of macrophages into the damaged nerve through a variety of mechanisms.

Activated macrophages play a primary role in removing myelin and axonal debris in the injured nerve. They infiltrate into the lesion site within the first 2 days and spread throughout the distal portion of the nerve beginning by Day 4, peak by 14 days and decrease thereafter (Muller and Minwegen, 1987; Avellino *et al.*, 1995). We show that although macrophages in the injured sciatic nerve express mainly cPLA$_2$ GIVA, some of these cells also express

iPLA$_2$ GVIA. Our current data shows that lack of cPLA$_2$ GIVA and/or iPLA$_2$ GVIA activity leads to a marked reduction in the clearance of myelin and axonal debris after sciatic nerve injury. LPC produced by PLA$_2$s binds with high affinity to C-reactive protein and IgM antibodies leading to the activation of the complement pathway, which is crucial for phagocytosis by macrophages (De *et al.*, 2003; Rotshenker, 2003). In addition, LPC produced by iPLA$_2$ may act as an 'eat me' signal for macrophages, as has been shown in other experimental models (Lauber *et al.*, 2003). Therefore, cPLA$_2$ GIVA and iPLA$_2$ GVIA expressed in peripheral nerves during Wallerian degeneration may also promote the phagocytic activity of macrophages.

One striking finding we observed is that the lack of iPLA$_2$ resulted in a notable accumulation of macrophages within the nerve at 21 days after injury. This observation might suggest that, iPLA$_2$ may influence the efflux of macrophages from the nerve at later stages of Wallerian degeneration. Previous studies have demonstrated that iPLA$_2$, but not cPLA$_2$, hydrolyzes docosahexaenoic acid (DHA) from phospholipids (Strokin *et al.*, 2003). DHA is the precursor of neuroprotectin D1 and resolvin D, two newly identified lipid mediators that have been shown to play an important role in the resolution of the inflammatory response (Schwab *et al.*, 2007). Therefore, iPLA$_2$ could be involved via these mechanisms in the clearance of macrophages from the injured nerve at late stages of Wallerian degeneration.

We also show that, the impaired Wallerian degeneration associated with the lack of cPLA$_2$ and iPLA$_2$ activity also delays axon regeneration and target re-innervation. The delay in axon regeneration may be due to the poor clearance of myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp), two axon growth inhibitors found in peripheral nerve myelin. Our results support earlier work by Schafer and co-workers (Schafer *et al.*, 1996) showing direct evidence for the effects of MAG in retarding axon regeneration in the WldS mouse, in which myelin clearance during Wallerian degeneration is severely impaired. Interestingly, after spinal cord injury where Wallerian degeneration is markedly slow, macrophages do not express cPLA$_2$ GIVA, and only a small population express iPLA$_2$ GVIA (unpublished results). Therefore, strategies that upregulate the expression of these intracellular PLA$_2$s may help speed the breakdown and clearance of myelin in the injured CNS and convert the non-permissive CNS white matter environment into a permissive one.

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