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ORIGINAL PAPER

Collagen VI regulates peripheral nerve regeneration by modulating macrophage recruitment and polarization

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Abstract Macrophages contribute to peripheral nerve regeneration and produce collagen VI, an extracellular matrix protein involved in nerve function. Here, we show that collagen VI is critical for macrophage migration and polarization during peripheral nerve regeneration. Nerve injury induces a robust upregulation of collagen VI, whereas lack of collagen VI in *Col6a1*$^{-/-}$ mice delays peripheral nerve regeneration. In vitro studies demonstrated that collagen VI promotes macrophage migration and polarization via AKT and PKA pathways. *Col6a1*$^{-/-}$ macrophages exhibit impaired migration abilities and reduced antiinflammatory (M2) phenotype polarization, but are prone to skewing toward the proinflammatory (M1) phenotype. In vivo, macrophage recruitment and M2 polarization are impaired in *Col6a1*$^{-/-}$ mice after nerve injury. The delayed nerve regeneration of *Col6a1*$^{-/-}$ mice is induced by macrophage deficits and rejuvenated by transplantation of wild-type bone marrow cells. These results identify collagen VI as a novel regulator for peripheral nerve regeneration by modulating macrophage function.

Keywords Collagen VI · Nerve regeneration · Macrophage · Migration · Polarization · Peripheral nerve

Introduction

Unlike the central nervous system, axons in the peripheral nervous system (PNS) have the ability to regenerate even after severe injury. Successful peripheral nerve regeneration is a process that requires the concerted interplay of glial cells, growth factors, cell adhesion molecules and extracellular matrix (ECM) proteins, as well as the recruitment of macrophages [21]. Macrophages are critical for the inflammatory response, a process that needs to be tightly controlled

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Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | ☐ LE | ☐ TYPESET
MS Code : ANEU-D-14-00635 | ☑ CP | ☑ DISK

to avoid excessive tissue damage after injury [31]. Following PNS injury, macrophages not only contribute to debris clearance, growth factor production and ECM remodeling in the distal nerve, but also stimulate regeneration near the axotomized neuronal cell bodies [20, 32, 34]. Leukemia inhibitory factor (LIF), interleukin (IL)-1α, IL-1β and monocyte chemoattractant protein-1 (MCP-1) have been identified as the major regulators for macrophage recruitment after peripheral nerve injury [36, 41, 45]. However, how these factors are modulated during macrophage recruitment remains elusive. Furthermore, additional factors for regulating macrophage migration after peripheral nerve injury need to be identified.

Macrophages exhibit remarkable plasticity and adopt pro- and antiinflammatory phenotypes (M1 and M2, respectively) in response to the stimulation of environmental signals [4, 5, 19]. Indeed, M1 and M2 macrophages exhibit distinct functions, where M1 macrophages stimulate an immune response, and M2 macrophages are immunosuppressive cells promoting tissue repair and remodeling [4, 6, 18, 29]. Interestingly, macrophages can undergo dynamic changes between M1 and M2 phenotypes, a process known as polarization skewing [32]. For example, when macrophages are stimulated with lipopolysaccharides (LPS) or interferon (IFN)-γ, they skew to an M1 phenotype characterized by high expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2. Conversely, macrophages are polarized to an M2 phenotype upon stimulation with IL-4, IL-10 or IL-13, a condition characterized by high expression of mannose receptor C type 1 (MRC1/CD206), arginase I (Arg-1) and peroxisomal proliferator activated receptor gamma (PPARγ) [4]. Polarization of macrophages toward the M2 phenotype in injury sites by local delivery of IL-4 promotes peripheral nerve regeneration [30]. However, the precise mechanisms governing macrophage polarization, especially in the peripheral nerve injury model, are still incompletely understood.

Collagen VI is a large ECM molecule made of three major genetically distinct chains, α1(VI), α2(VI) and α3(VI), which are encoded by *Col6a1*, *Col6a2* and *Col6a3* genes, respectively [5]. Although our previous studies demonstrated that collagen VI is an essential component of peripheral nerves required for proper nerve myelination and function [7], the role of collagen VI in peripheral nerve regeneration is completely unknown. M2 macrophages produce higher amounts of collagen VI than M1 macrophages [40]. Moreover, collagen VI enhances the adhesion of monocytes [40]. These findings raise the question whether collagen VI is required for macrophage activities, such as migration and polarization. Here, we show that collagen VI is critical for macrophage migration and M2 polarization via AKT and PKA pathways. As a result, peripheral nerve regeneration is strikingly impaired in collagen VI null ($Col6a1^{-/-}$) mice, where a targeted inactivation of the *Col6a1* gene blocks the assembly and secretion of collagen VI [2, 23]. These findings provide novel mechanistic data for macrophage activity and plasticity and demonstrate that collagen VI is a key regulator of PNS regeneration through modulation of macrophage function.

Materials and methods

Animals

$Col6a1^{+/+}$ (wild-type) and $Col6a1^{-/-}$ mice in the C57BL/6 background were used in this study [2, 23]. All in vivo experiments were performed in 6–7-month-old mice. Native collagen VI protein was purified from newborn mice as previously described [23]. Animal procedures were approved by the Ethics Committee of the University of Padua and authorized by the Italian Ministry of Health.

Surgical procedures

Mice were anesthetized with ketamine (100 mg/kg body weight) and xylazine (8 mg/kg body weight), and the right sciatic nerve was exposed and crushed with a liquid nitrogen-cooled Dumont forceps for 20 s, stopped for a 10 s interval and then subjected to a second crush at the same site. The crush site was about 45 mm from the tip of the third digit, which was labeled with India ink.

Macrophage depletion

Macrophages were depleted by i.p. injection of clodronate liposome (ClodronateLiposomes.com) in mice as reported previously [16]. Briefly, clodronate liposome (200 μl/mouse) was injected at 1, 3, 5 and 8 days post-crush to obtain macrophage-depleted mice. Control mice received an equal volume of PBS liposome at the same time points.

Bone marrow transplantation

The bone marrow transplantation was performed as described previously [12]. Briefly, bone marrow was harvested from 6- to 8-week-old wild-type mice by flushing the femurs and tibias with 2 % fetal bovine serum in phosphate-buffered saline. Cells (2×10^6) were intravenously injected through the tail vein into lethally irradiated (10 Gy) 4-month-old wild-type and $Col6a1^{-/-}$ mice. Sciatic nerve injury was performed 6 weeks after the transplantation.

Functional tests

Prior to and after crush, nontoxic paint was applied to the hindpaws, and the mice were allowed to walk on a white

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | ☐ LE | ☐ TYPESET
MS Code : ANEU-D-14-00635 | ☑ CP | ☑ DISK

paper. From the footprint gait, the parameters of print length (the distance between the heel and the third toe, abbreviated as PL) and toe spread (the distance from the first toe to the fifth toe, abbreviated as TS) from both the normal side (N) and experimental side (E) were recorded. The sciatic functional index (SFI) was calculated with the following formula: SFI = 118.9 [(ETS−NTS)/NTS] −51.2 [(EPL−NPL)/NPL] −7.5 as previously reported [22, 52]. Analysis of the toe spread reflex and toe pinch was carried out as previously described [43].

Histology

Prior to injury and at 7, 21 and 29 days following crush injury, 3 mice of each group were perfused with 4 % paraformaldehyde, the 3-mm distal portion of sciatic nerves was dissected into 1-mm segments and postfixed in 2 % glutaraldehyde for 24 h at 4 °C. Samples (3 mm distal to the site of injury) were osmicated in 2 % osmium tetroxide for 2 h at room temperature, dehydrated in ascending acetone and embedded in Epon E812 resin (Sigma). Semithin sections (0.5 μm) were cut using an Ultracut 200 microtome (Leica) and stained with alkaline toluidine blue. Myelinated axon numbers were analyzed on eight sections per sciatic nerve. For electron microscopy, ultrathin sections (80 nm) were cut, mounted on copper grids, and stained with uranyl citrate and lead citrate. Grids were observed and photographed on an FEI Tecnai 12 transmission electron microscope. Phagocytic macrophages were identified by the "foamy" morphology, which is induced by the presence of end products of myelin/lipid degradation, as described in previous studies [26].

Matrigel plug assay

A total of 500 μl growth factor-reduced Matrigel (Gibco) supplemented with PBS, 2 % FBS, purified collagen VI (500 ng/ml), purified collagen I (Sigma, 500 ng/ml) or MCP-1 (ImmunoTools, 10 ng/ml) was injected subcutaneously into wild-type and *Col6a1*$^{-/-}$ mice. After 7 days, the Matrigel plug was harvested and processed for immunofluorescence.

Cell cultures

The J774 macrophage cell line was purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10 % fetal bovine serum (FBS, Gibco), 0.2 M L-glutamine (Invitrogen) and 1:100 penicillin-streptomycin (Invitrogen). J774 cells were treated with purified native collagen VI for further studies. Primary bone marrow-derived macrophages (BMDMs) and peritoneal macrophage (PMs) were isolated and cultured as previously described with minor modifications [8, 25, 52]. Briefly, for BMDM isolation and culture, 2–3-month-old mice were killed, and bone marrow cells collected by flushing both femurs and tibias with culture medium. Red blood cells were removed using a lysis buffer (150 mM NH_4Cl, 0.1 mM Na_2EDTA and 1 mM $KHCO_3$, pH 7.2) for 10 min at room temperature. The remaining cells were then differentiated with 30 ng/ml M-CSF (ImmunoTools) in DMEM containing 20 % FBS, 0.2 M L-glutamine and 1:100 penicillin-streptomycin for 1 week until the cells reached confluence. For PM isolation and culture, 3 % thioglycolate broth (Sigma) was injected intraperitoneally to induce peritonitis in 2–3-month-old mice. Three days later, peritoneal cells were collected and cultured in DMEM containing 10 % FBS, 0.2 M L-glutamine and 1:100 penicillin-streptomycin. Two hours later, nonadherent cells were removed by washing with PBS, and adherent macrophages were used for further studies. BMDMs and PMs were differentiated into the M2 or M1 phenotype with 20 ng/ml IL-4 (ImmunoTools) or 5 ng/ml LPS (Sigma), respectively, for 24 h.

Migration assay

Macrophage migration was assessed using transwell inserts with 5-μm pores (Millipore). Briefly, J774 macrophages (2×10^4 cells per well) were seeded into the upper chamber of a transwell filter with DMEM. The same culture medium and purified collagen VI (0.5 or 1 μg/ml), purified collagen I (1 μg/ml) or MCP-1 (10 ng/ml) were added to the lower chamber. When indicated, cells were treated with AKTi (Sigma, 10 μM) or H89 (Sigma, 30 μM). Cells were allowed to migrate for 8 h at 37 °C and 5 % CO_2. After being fixed and stained with ethanol and 0.05 % crystal violet (Sigma), the migrated cells were counted in eight different areas under a light microscope.

Scratch assay

A wound was made in confluent monolayers of J774 cells grown on six-well cell culture plates by scraping with a sterile 200-μl pipette tip. The cells were gently rinsed with PBS and further cultured in the presence or absence of purified collagen VI (1 μg/ml), purified collagen I (1 μg/ml), MCP-1 (10 ng/ml), AKTi (10 μM) or H89 (30 μM). Images of the cultures were taken immediately after scratching and after 8 h. The migration distances of the macrophages were measured and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics).

RNA isolation and real-time RT-PCR

Total RNA from mouse sciatic nerves (3 mm distal to injury site) was isolated using Trizol reagent (Life Technologies)

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | ☐ LE | ☐ TYPESET
MS Code : ANEU-D-14-00635 | ☑ CP | ☑ DISK

following the manufacturer's instructions; 200 ng of total RNA was used to make cDNA using the Superscript II kit (Invitrogen). Quantitative PCR was carried out using the LightCycler 480 system (Roche). The expression level of each gene was calculated by comparing with the *Gapdh* housekeeping gene. Primers used in this study are shown in Supplementary Table 1.

Immunofluorescence

After mice had been perfused with 4 % paraformaldehyde, a 3-mm length of sciatic nerve distal to the crush site was removed and postfixed for 4 h at 4 °C. Tissues or Matrigel plugs were then transferred into 30 % sucrose overnight for cryoprotection. Samples of 10 μm were cut in a cryostat (Leica). After blocking with 10 % goat serum for 1 h, sections were incubated with primary antibodies (1:200 dilution) for 2 h at room temperature or overnight at 4 °C. Primary antibodies against the following proteins were used: α3(VI) collagen (guinea pig polyclonal, a gift of Raimund Wagener, Cologne, Germany) [15]; β-III tubulin (mouse monoclonal, Sigma); CD68, F4/80 (rat monoclonal, AbD Serotec); CD206 (rabbit polyclonal, Abcam); MAG (rabbit monoclonal, Cell Signaling). The samples were then transferred to secondary antibodies (1:200 dilution) and Hoechst 33258 (Sigma) for 1 h at room temperature. The following secondary antibodies were used: anti-rat CY3, anti-rabbit CY3 and anti-guinea-pig CY2 (Jackson Immunoresearch). After washing three times in PBS, slides were mounted using 80 % glycerol.

Western blotting

Mice were killed by cervical dislocation, and sciatic nerves (3 mm distal to the crush site) were removed and frozen in nitrogen immediately. The tissues or cells were homogenized in lysis buffer (Millipore) with phosphatase inhibitors (Sigma) and protease inhibitors (Roche, Basel, Switzerland). The protein concentration was determined by BCA assay (Thermo Sciemtific). Samples of 20 μg protein were applied to SDS-PAGE gels (Invitrogen) and blotted onto a PDVF membrane (Millipore). Membranes were incubated with primary antibodies (1:1,000 dilution) overnight at 4 °C. Primary antibodies against the following proteins were used for Western blot analysis: α1(VI) collagen, Arg-1, CD206, iNOS (rabbit polyclonal, Santa Cruz Biotechnology); β-actin (mouse monoclonal, Sigma); AKT, phospho-PKA, PKA (rabbit polyclonal, Cell Signaling); phospho-AKT, COX-2, MAG, PPARγ (rabbit monoclonal, Cell Signaling); CD16 (rabbit monoclonal, Abcam); CD206 (rabbit polyclonal, Abcam); CD68 (rat monoclonal, AbD Serotec). After washing three times with TBST, membranes were incubated with HRP-conjugated secondary antibodies (1:1,000 dilution; Amersham Bioscience) for 1 h at room temperature. Detection was by chemiluminescence (Pierce). The panels show representative images of two separate protein extracts derived from two different mice. Densitometric quantification was performed by Image-Pro Plus 6.0 software (Media Cybernetics).

Statistical analysis

Data are represented as mean ± SEM. Statistical analysis of data was carried out using Student's *t* test, except for the analysis of the toe spread reflex in PBS- and clodronate-liposome-treated mice, where the chi-square test was used, and the analysis of collagen VI mRNA expression after injury, where one-way ANOVA followed by post hoc tests was used. $P < 0.05$ was considered as a significant difference.

Results

Expression of collagen VI is increased after sciatic nerve crush injury

To explore the role of collagen VI in PNS regeneration, we first examined whether collagen VI expression is upregulated upon sciatic nerve crush injury in adult mice. Real-time RT-PCR showed that the levels of *Col6a1* and *Col6a3* transcripts were increased at 7 and 14 days post-injury, whereas the levels of *Col6a2* transcripts started to increase within 3 days after sciatic nerve crush, and the expression of all three mRNAs reached a peak between 3 and 7 days post-injury (Fig. 1a–c). Western blot analysis for α1(VI) and α2(VI) chains (Fig. 1d) and immunofluorescence for α3(VI) chain (Fig. 1e, f) showed that the protein levels for collagen VI were also increased between 3 and 7 days post-injury. Taken together, these data indicate that crush injury of the sciatic nerve induces a robust upregulation of both mRNA and protein levels of collagen VI, pointing to a potential role for this molecule during PNS regeneration.

Collagen VI is required for peripheral nerve regeneration

Wallerian degeneration is a process that includes the breakdown of axons and phagocytosis of damaged axons and myelin debris after injury, which is strictly required for axon regeneration [36]. We therefore first examined Wallerian degeneration in collagen VI-deficient mice. Toluidine blue staining and electron microscopy showed that at 7 days post-injury sciatic nerves from wild-type mice had advanced signs of myelin breakdown and a high incidence of phagocytic macrophages. However, both of these features were noticeably lower in *Col6a1*$^{-/-}$ mice

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | LE | TYPESET
MS Code : ANEU-D-14-00635 | CP | DISK

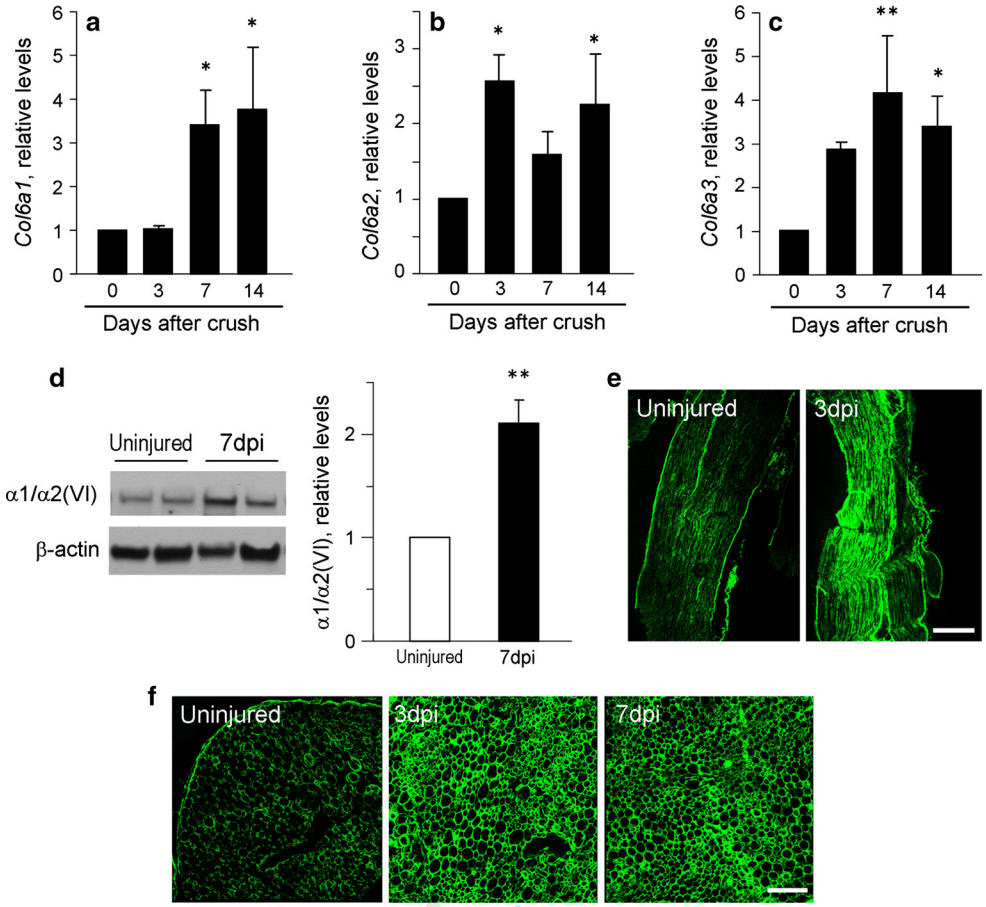

Fig. 1 Collagen VI expression is enhanced upon peripheral nerve crush. Real-time RT-PCR for *Col6a1* (**a**), *Col6a2* (**b**) and *Col6a3* (**c**) in uninjured (0) or injured sciatic nerves at 3, 7 and 14 days post-crush ($n = 3$–5). There was a statistically significant difference among groups as determined by one-way ANOVA analysis of *Col6a1* [$F(3,13) = 3.739$, $P = 0.039$], *Col6a2* [$F(3,13) = 3.506$, $P = 0.046$] and *Col6a3* [$F(3,13) = 4.600$, $P = 0.021$]. A post hoc test revealed that the relative levels of *Col6a1* or *Col6a3* were statistically significantly increased in injured nerves at 7 dpi (3.46 ± 0.77, $P = 0.035$, or 4.15 ± 1.29, $P = 0.004$) and 14 dpi (3.76 ± 1.44, $P = 0.018$, or 3.38 ± 0.68, $P = 0.033$), but not at 3 dpi (1.04 ± 0.05, $P = 0.973$, or 2.85 ± 0.17, $P = 0.087$), when compared to uninjured nerves, and the relative levels of *Col6a2* were statistically significantly increased in injured nerves at 3 dpi (2.55 ± 0.35, $P = 0.014$) and 14 dpi (2.24 ± 0.68, $P = 0.028$), but not at 7 dpi (1.59 ± 029, $P = 0.271$), when compared to uninjured nerves. There was no statistically significant difference among the 3-, 7- and 14-dpi groups for *Col6a2* ($P = 0.415$) and *Col6a3* ($P = 0.639$). *Col6a1* was significantly increased in injured nerves at 14 dpi compared to 3 dpi ($P = 0.043$), but no significant differences were seen between the 7- and 14-dpi ($P = 0.758$) and the 3- and 7-dpi ($P = 0.072$) groups. **d** *Left panel* Western blot analysis for α1/α2(VI) in uninjured sciatic nerves or injured nerves at 7 days post-crush. *Right panel* Densitometric quantification of α1/α2(VI) vs. actin as determined by three independent Western blot experiments ($n = 4$; $^{**}P < 0.01$). **e** Immunofluorescence for α3(VI) in longitudinal sections of uninjured sciatic nerves and injured nerves at 3 days post-crush. *Scale bar* 250 μm. **f** Immunofluorescence for α3(VI) in cross sections of uninjured sciatic nerves and injured nerves at 3 and 7 days post-crush. *Scale bar* 50 μm. *dpi* Days post-injury

(Supplementary Fig. S1a, d). Quantitative analysis confirmed that *Col6a1*$^{-/-}$ nerves had more myelinated axons and fewer phagocytic macrophages than wild-type nerves at 7 days post-injury (Supplementary Fig. S1b, c). In keeping with the concept that clearance of myelin debris from injured nerves is necessary for PNS regeneration [14, 47], more myelin was present in *Col6a1*$^{-/-}$ nerves than in wild-type nerves at 7 days post-injury (Supplementary Fig. S1a, d). Axonal growth inhibitors, such as myelin-associated glycoprotein (MAG), are usually present in myelin debris after nerve injury [39, 51]. Immunofluorescence and Western blot analysis revealed that MAG reactivity was higher in *Col6a1*$^{-/-}$ nerves than wild-type nerves at 7 days post-injury (Supplementary Fig. S1e, f). These data support the potential role of collagen VI in Wallerian degeneration following injury.

We then performed experiments to assess whether the inhibited Wallerian degeneration of *Col6a1*$^{-/-}$ mice influences PNS regeneration. First, we measured the sciatic functional index to evaluate the recovery of sensory motor coordination [24] in mice of both genotypes. As shown in Fig. 2a, the sciatic functional index score was not different

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | LE | TYPESET
MS Code : ANEU-D-14-00635 | CP | DISK

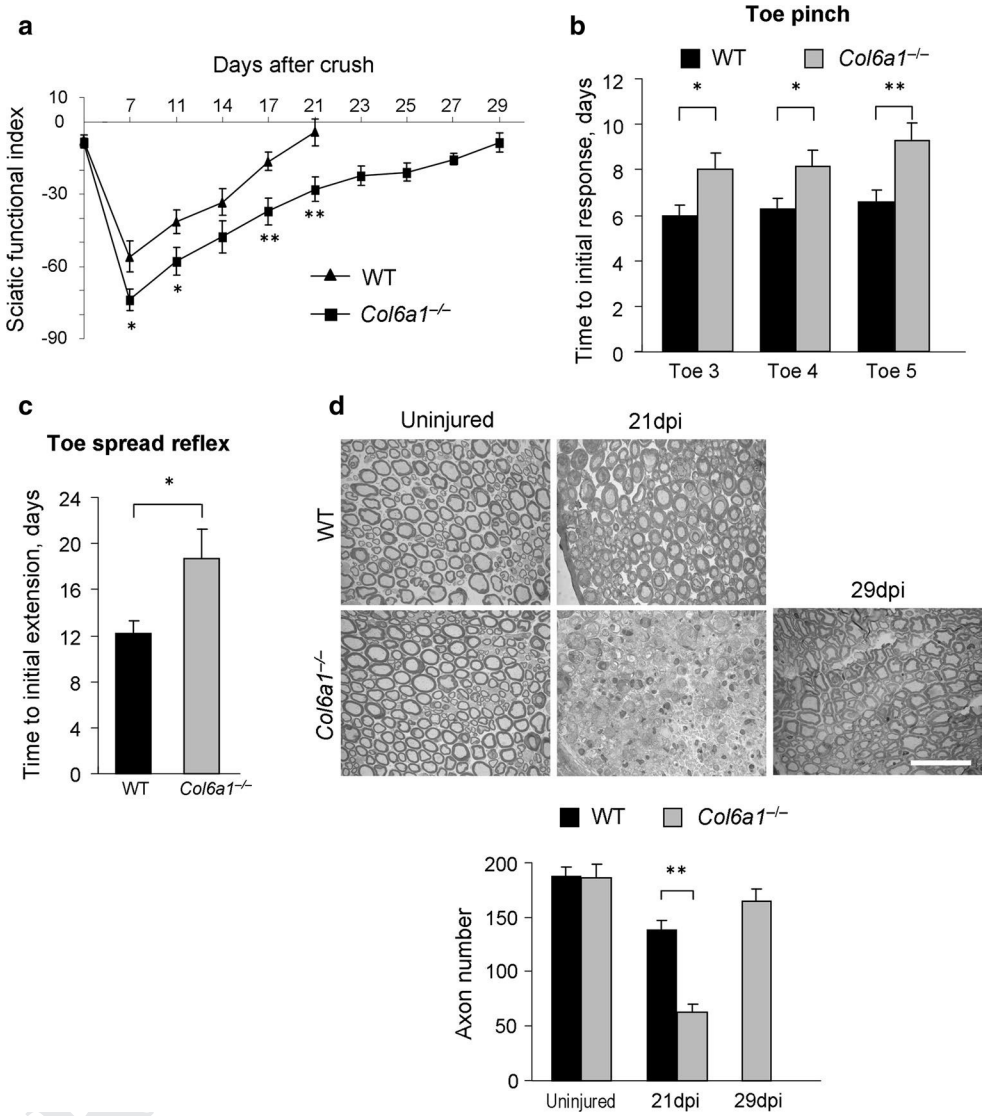

Fig. 2 Lack of collagen VI impairs peripheral nerve regeneration. **a** Quantification of sensory-motor function of wild-type and *Col6a1*$^{-/-}$ mice by analyzing the sciatic functional index from the footprint track before crush and at 7, 11, 14, 17, 21 and 29 days post-crush ($n = 7$; $^{*}P < 0.05$; $^{**}P < 0.01$). **b** Quantification of sensory function of wild-type and *Col6a1*$^{-/-}$ mice after sciatic nerve crush by recording the initial response time (day post-injury) to the pinch using forceps in the digits 3, 4 and 5 ($n = 7$; $^{*}P < 0.05$; $^{**}P < 0.01$). **c** Quantification of motor function of wild-type and *Col6a1*$^{-/-}$ mice after sciatic nerve crush by recording the initial extension time (day post-injury) to toe spreading reflex ($n = 7$; $^{*}P < 0.05$). **d** Representative images of toluidine blue staining and morphometric analysis of the myelinated axon number in cross sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 21 and 29 days post-crush ($n = 3$; $^{**}P < 0.01$). *Scale bar* 40 μm. *dpi* days post-injury, *WT* wild type

between wild-type and *Col6a1*$^{-/-}$ mice before nerve crush. In contrast, the sciatic functional index of *Col6a1*$^{-/-}$ mice was significantly lower than that of wild-type mice at 7 days after injury. This parameter remained significantly lower in collagen VI-deficient mice during the following time points, and a complete functional recovery was observed at 21 and 29 days post-injury in wild-type and *Col6a1*$^{-/-}$ mice, respectively (Fig. 2a). To measure sensory functions, we recorded the response to toe pinch in digits 3, 4 and 5 of the crushed hindlimb because they are the main digits innervated by nerves for sensory functions [26]. The time to initial response to the stimuli after sciatic nerve crush was significantly longer in *Col6a1*$^{-/-}$ mice than wild-type mice (Fig. 2b). Next, we utilized the toe spread reflex to evaluate motor function and found that the time to initial toe extension after nerve injury was significantly increased in *Col6a1*$^{-/-}$ mice when compared to wild-type mice (Fig. 2c). Toluidine blue staining showed that the number of myelinated axons was significantly lower in *Col6a1*$^{-/-}$ nerves than in wild-type nerves at 21 days post-injury, whereas there was no difference between the two genotypes in uninjured conditions (Fig. 2d). In agreement with the sciatic functional index, the number of myelinated axons was almost completely restored at 21 days post-injury in wild-type mice, while this required 29 days in *Col6a1*$^{-/-}$ mice (Fig. 2d). Altogether, these findings indicate that lack of collagen VI delays peripheral nerve regeneration after injury.

Collagen VI stimulates macrophage migration in vitro and in vivo

In the PNS, macrophages are critical for the removal of debris and contribute to nerve regeneration [13, 51]. To determine whether collagen VI is critical for macrophage activities, we performed in vitro and in vivo experiments to analyze macrophage migration. Transwell assay showed that

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | LE | TYPESET
MS Code : ANEU-D-14-00635 | CP | DISK

addition of purified collagen VI to the culture medium in the lower chambers at the concentration of 1 μg/ml significantly increased the number of macrophages that had migrated (Fig. 3a and Supplementary Fig. S2a). Scratch assay revealed that collagen VI promotes macrophage motility after scratching, as demonstrated by the markedly enhanced migration distance when macrophages were treated with collagen VI (Fig. 3b and Supplementary Fig. S2b). To investigate the in vivo chemoattractant ability of collagen VI, we used a Matrigel plug assay to examine macrophage density in the Matrigel implanted subcutaneously into wild-type mice. Immunofluorescence for CD68 and F4/80 showed that both these macrophage markers were markedly increased in wild-type mice treated with Matrigel plugs supplemented with purified collagen VI compared to mice treated with PBS-supplemented Matrigel plugs (Fig. 3c and Supplementary Fig. S2c). In addition, we utilized collagen I and MCP-1 as negative and positive controls, respectively, and found that MCP-1, but not collagen I, significantly enhanced macrophage migration in transwell, scratch and Matrigel plug assays (Fig. 3a–c and Supplementary Fig. S2a, b). Next, we used a different experimental setting, where Matrigel plugs supplemented with 2 % FBS were subcutaneously injected in wild-type and *Col6a1*$^{-/-}$ mice. Immunofluorescence for the F4/80 marker showed that the macrophage migration capability was dramatically impaired in *Col6a1*$^{-/-}$ mice compared to wild-type animals (Fig. 3d), suggesting that in addition to as a chemokine itself, collagen VI is required for other factors inducing macrophage migration.

It has been demonstrated that the AKT and PKA pathways are necessary for macrophage migration [10, 11]. We thus investigated whether collagen VI-induced macrophage migration is regulated by these signals. Western blot analysis showed that the addition of collagen VI to in vitro macrophages promoted AKT and PKA phosphorylation (Supplementary Fig. S3a, b). Transwell assay demonstrated that the collagen VI-induced increase in the number of migrated macrophages was inhibited by pretreatment with AKT inhibitor (AKTi) or H89, a PKA inhibitor (Fig. 3a and Supplementary Fig. S2a). Moreover, the scratch assay revealed that pretreatment with AKTi or H89 decreased the collagen VI-induced macrophage migration distance (Fig. 3b and Supplementary Fig. S2b). Taken together, these data indicate that collagen VI promotes macrophage migration by regulating the AKT and PKA pathways.

Ablation of collagen VI leads to impaired macrophage recruitment to the injured nerve

Given the robust chemoattractant activity of collagen VI for macrophages, we further investigated whether the delayed myelin clearance and PNS regeneration are the result of impaired macrophage recruitment in *Col6a1*$^{-/-}$ injured nerves. Immunofluorescence showed that more CD68- and F4/80-positive macrophages were present in the injured nerves of wild-type mice than *Col6a1*$^{-/-}$ mice (Fig. 4a–c). Western blot analysis confirmed that although CD68 was enhanced in both genotypes at 7 days post-injury, the CD68 levels of injured *Col6a1*$^{-/-}$ nerves were significantly lower than those of injured wild-type nerves (Fig. 4d). These data indicate that lack of collagen VI impairs macrophage accumulation in injured nerves.

Chemokines and cytokines are important mediators of the immune response. Among them, IL-1β and MCP-1 are two prominent regulators of macrophage recruitment in injured peripheral nerves [36, 41, 45]. We therefore examined whether the impaired macrophage recruitment in *Col6a1*$^{-/-}$ mice after injury was paralleled by a lower abundance of these two inflammatory regulators. Real-time RT-PCR revealed that although the expression of IL-1β and MCP-1 mRNA was upregulated in both wild-type and *Col6a1*$^{-/-}$ nerves at 1 day after crush, the levels of the two transcripts were significantly lower in injured *Col6a1*$^{-/-}$ nerves compared to injured wild-type nerves (Supplementary Fig. S4a, b). These results suggest that in addition to its chemoattractant activity for macrophage migration, collagen VI also affects other inflammatory mediators in injured peripheral nerves.

Collagen VI is critical for macrophage polarization

Peripheral nerve regeneration not only depends on macrophage density, but also requires macrophage polarization toward the M2 phenotype [30]. To investigate the potential role of collagen VI in macrophage polarization, we isolated primary bone marrow-derived macrophages (BMDMs) and peritoneal macrophages (PMs) from wild-type and *Col6a1*$^{-/-}$ mice and differentiated them toward the M2 and M1 phenotypes with IL-4 and LPS, respectively. Western blot analysis for M2 markers showed that the protein levels of Arg-1, CD206 and PPARγ were increased in wild-type, but not in *Col6a1*$^{-/-}$ BMDMs upon stimulation with IL-4 (Fig. 5a). Similar results were found in PMs, where Arg-1 and PPARγ levels were upregulated in wild-type cells upon stimulation of IL-4, whereas this enhancement was prevented in *Col6a1*$^{-/-}$ cells (Fig. 5b). Furthermore, the defective response of *Col6a1*$^{-/-}$ PMs was reversed when cells were cultured in the presence of purified collagen VI (Fig. 5b). These results indicate that collagen VI is required for macrophage M2 polarization.

Since collagen VI expression is reduced when macrophages are subjected to M1 stimuli [40], we hypothesized that this reduction may be essential for M1 polarization. Upon LPS stimulation, *Col6a1*$^{-/-}$ PMs displayed a marked enhancement of COX-2, a M1 marker, when compared to wild-type PMs (Fig. 5c). A similar response was found in BMDMs, since upon LPS stimulation *Col6a1*$^{-/-}$ cells exhibited higher

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | ☐ LE | ☐ TYPESET
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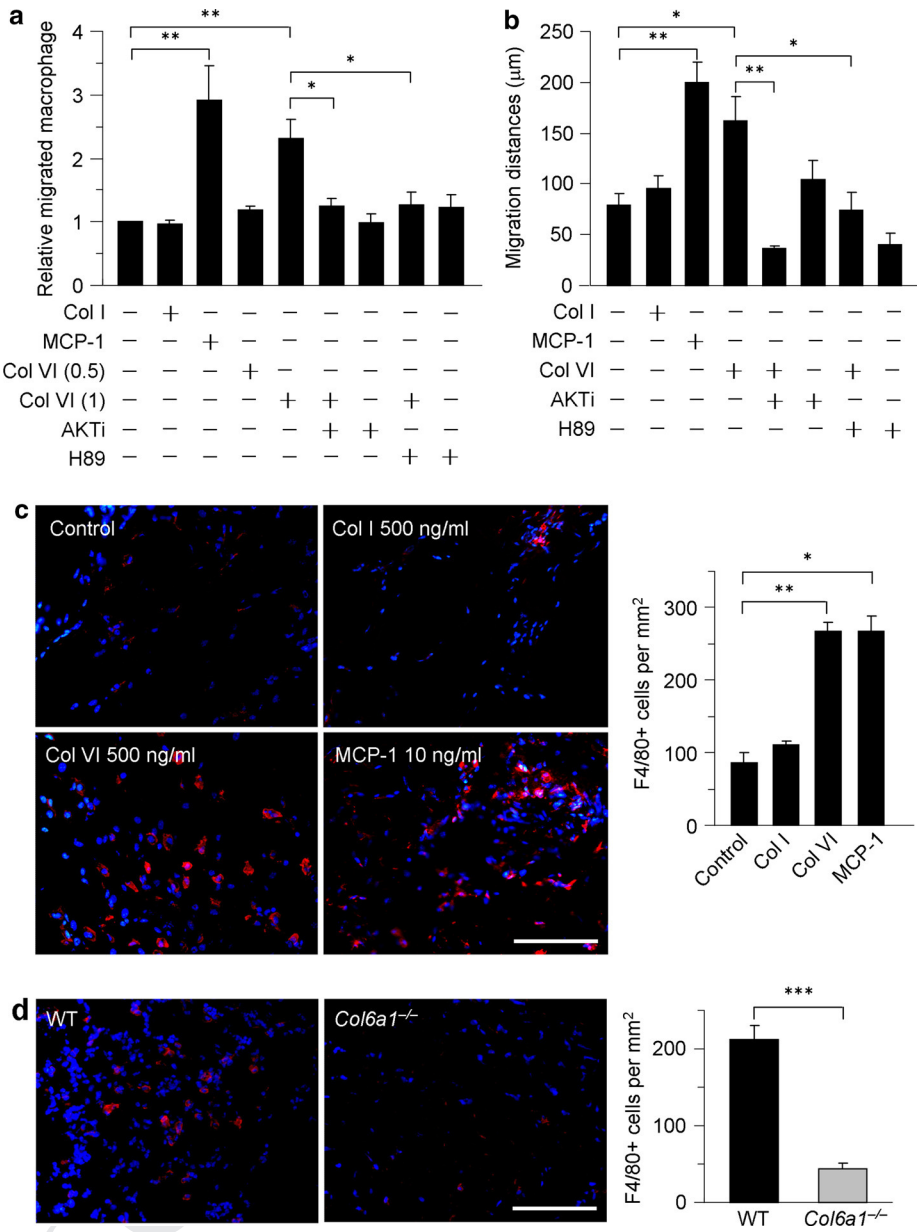

Fig. 3 Collagen VI promotes in vitro and in vivo macrophage migration. **a** Quantification of migrated J774 macrophages in transwell migration assays upon treatment with PBS (control), collagen VI (0.5 or 1 μg/ml), collagen I or MCP-1. AKTi or H89 was added where indicated ($n = 3$–4; $^*P < 0.05$; $^{**}P < 0.01$). **b** Quantification of mean migration distances of J774 macrophages upon treatment with PBS (control), collagen VI (1 μg/ml), collagen I or MCP-1 and subjected to a scratch assay. AKTi or H89 was added where indicated. Cells were analyzed 8 h after the scratch ($n = 3$–4; $^*P < 0.05$; $^{**}P < 0.01$). **c** *Left and middle panels* Representative images of immunofluorescence for F4/80 in growth factor-reduced Matrigel plugs supplemented with PBS, collagen VI, collagen I or MCP-1 subcutaneously injected into wild-type mice. *Scale bar* 100 μm. *Right panel* Quantitative analysis of migrated F4/80-positive macrophages in Matrigel plugs ($n = 3$; $^*P < 0.05$; $^{**}P < 0.01$). **d** *Left and middle panels* Representative images of immunofluorescence for F4/80 in growth factor-reduced Matrigel plugs supplemented with 2 % FBS subcutaneously injected into wild-type (*left*) and *Col6a1*$^{-/-}$ (*middle*) mice. *Scale bar* 100 μm. *Right panel* Quantitative analysis of migrated F4/80-positive macrophages in Matrigel plugs ($n = 3$; $^{***}P < 0.001$). *AKTi* AKT inhibitor, *Col I* Collagen I, *Col VI* collagen VI, *WT* wild type

enhancement of several M1 markers, such as iNOS, CD16 and COX-2, when compared to wild-type cells (Fig. 5d, e). Of note, these enhancements in *Col6a1*$^{-/-}$ cells were partially rescued when cells were cultured in the presence of purified collagen VI (Fig. 5d, e). These results indicate that collagen VI inhibits macrophage M1 polarization.

To further confirm the effect of collagen VI in macrophage polarization, we cultured J774 macrophages and

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | LE | TYPESET
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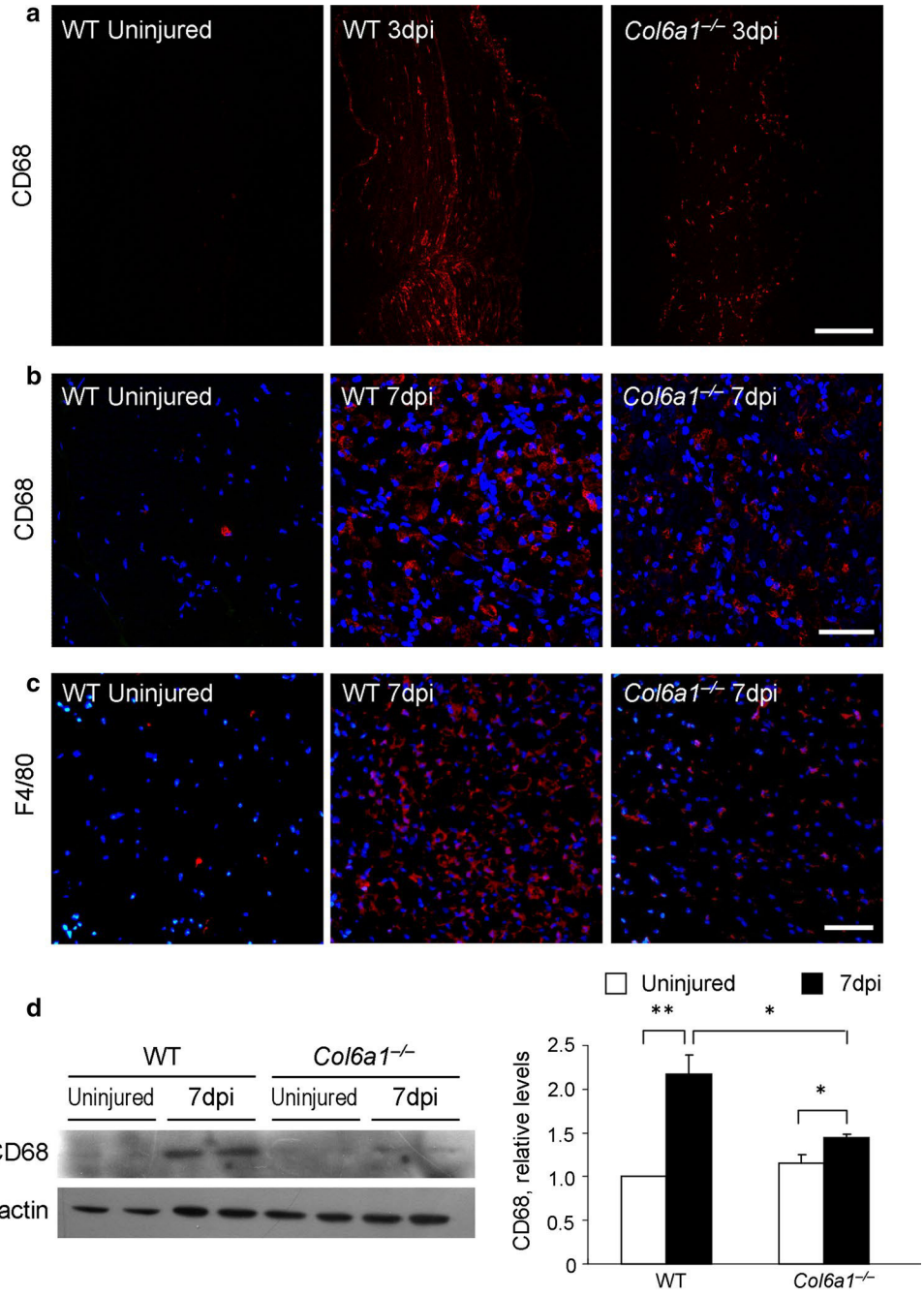

Fig. 4 Lack of collagen VI leads to impaired of macrophage recruitment into injured nerves. **a** Immunofluorescence for CD68 in longitudinal sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 3 days post-crush. *Scale bar* 250 μm. **b** Immunofluorescence for CD68 in cross sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. *Scale bar* 50 μm. **c** Immunofluorescence for F4/80 in cross sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. *Scale bar* 50 μm. **d** *Left panel* Western blot analysis for CD68 in sciatic nerves of wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. *Right panel* Densitometric quantification of CD68 vs. actin as determined by three independent Western blot experiments. Values for uninjured wild-type nerves were arbitrarily set to 1 ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$). *dpi* days post-injury, *WT* wild type

wild-type PMs in the absence or presence of purified collagen VI. Addition of collagen VI enhanced Arg-1 levels and reduced the levels of CD16 and iNOS in J774 macrophages (Supplementary Fig. S5a–c). Moreover, Western blot analysis for Arg-1 and PPARγ in PMs showed that both of these M2 markers were significantly increased upon treatment with purified collagen VI (Supplementary Fig. S5d, e). Taken together, these findings indicate that collagen VI promotes macrophage M2 polarization and exhibits an inhibitory effect on macrophage M1 polarization.

AKT and PKA are two key mediators of signaling pathways involved in macrophage polarization [3, 27, 38]. We thus evaluated whether collagen VI-induced macrophage polarization involves the activation of AKT and PKA signals. Western blot analysis showed that the phosphorylation of both AKT and PKA was increased upon stimulation with IL-4 in wild-type, but not in *Col6a1*$^{-/-}$ PMs (Supplementary Fig. S6a). Addition of collagen VI to BMDMs enhanced CD206 levels, which were inhibited by AKTi and H89 (Supplementary Fig. S6b). Moreover, immunofluorescence on J774 macrophages showed that collagen

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | LE | TYPESET
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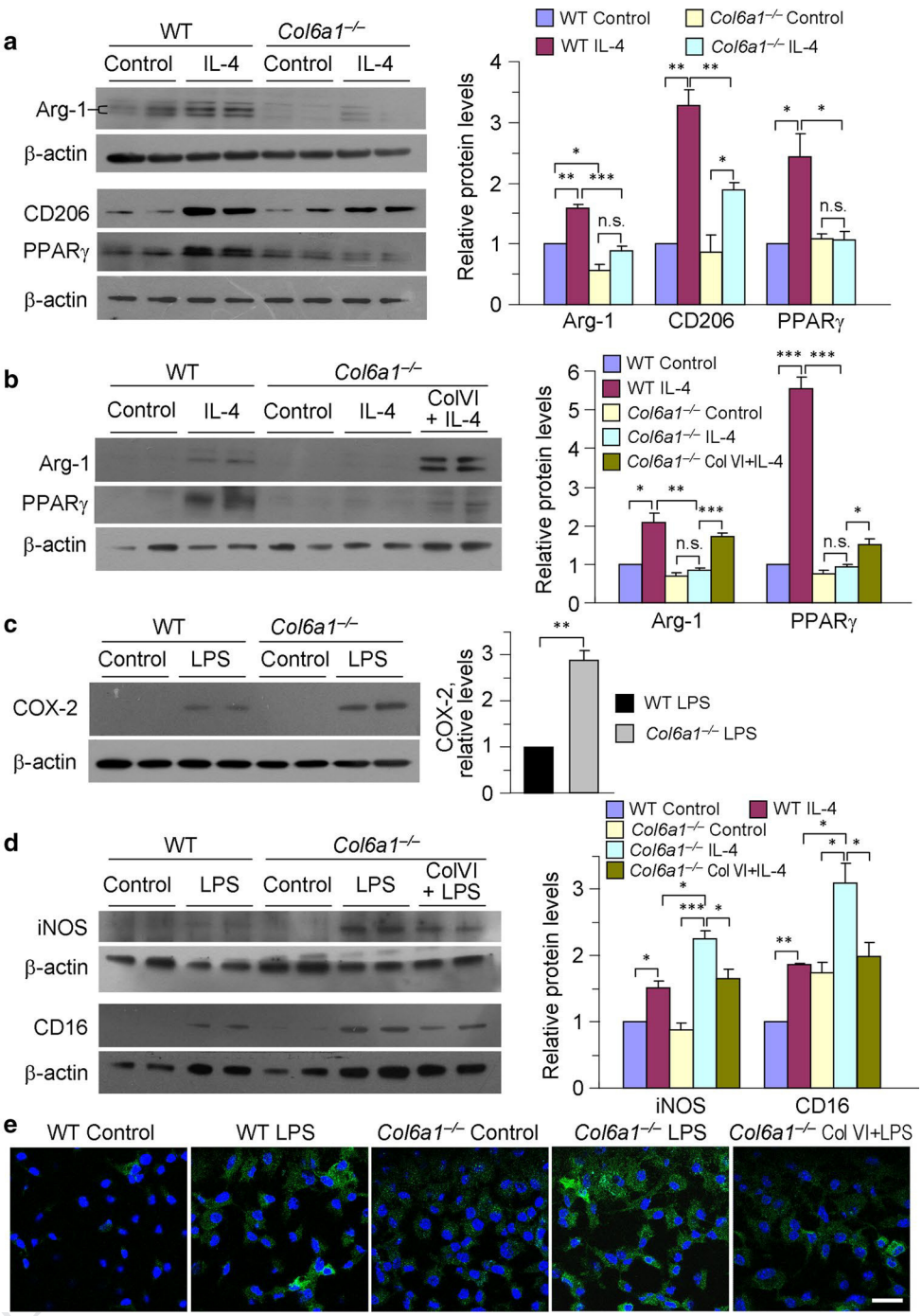

VI-promoted PPARγ expression was attenuated by AKTi and H89 (Supplementary Fig. S6c). These data support a role for the AKT and PKA pathways in modulating collagen VI-related macrophage M2 polarization.

Lack of collagen VI impairs macrophage M2 polarization in vivo after peripheral nerve injury

Next, we investigated the in vivo role of collagen VI in macrophage polarization in injured nerves. CD16 protein levels were higher in *Col6a1*$^{-/-}$ nerves than in wild-type nerves at 7 days post-crush (Fig. 6a). Furthermore, the expression of M2 marker genes *Arg1* and *Mrc1* was upregulated in wild-type nerves, but not in *Col6a1*$^{-/-}$ nerves at 7 days after crush (Fig. 6b, c). Immunofluorescence for CD206 showed that the amount of M2 macrophages in *Col6a1*$^{-/-}$ nerves was dramatically lower than in wild-type nerves at 7 days post-injury (Fig. 6d). Western blot analysis confirmed that the levels of CD206 were significantly increased in wild-type nerves, but not in *Col6a1*$^{-/-}$ nerves, at 7 days after crush when

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | LE | TYPESET
MS Code : ANEU-D-14-00635 | CP | DISK

◄ **Fig. 5** Effect of collagen VI on macrophage polarization. **a** *Left panel* Western blot analysis for Arg-1, CD206 and PPARγ in wild-type and *Col6a1*$^{-/-}$ BMDMs under control conditions or following induction with IL-4. *Right panel* Densitometric quantification of Arg-1 vs. actin, CD206 vs. actin and PPARγ vs. actin, as determined by three independent Western blot experiments. Values for the wild-type control group were arbitrarily set to 1 ($n = 4$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; *n.s.* not significant). **b** *Left panel* Western blot analysis for Arg-1 and PPARγ in wild-type, *Col6a1*$^{-/-}$ and collagen VI-coated (5 μg/cm^2) *Col6a1*$^{-/-}$ PMs under control conditions or following induction with IL-4. *Right panel* Densitometric quantification of Arg-1 vs. actin and PPARγ vs. actin, as determined by three independent Western blot experiments. Values for the wild-type control group were arbitrarily set to 1 ($n = 3$–4; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; *n.s.* not significant). **c** *Left panel* Western blot analysis for COX-2 in wild-type and *Col6a1*$^{-/-}$ PMs under control conditions or following induction with LPS. *Right panel* Densitometric quantification of COX-2 vs. actin, as determined by three independent Western blot experiments. Only LPS-induced COX-2 levels were calculated because of the extremely low COX-2 levels at baseline. Values for the wild-type LPS group were arbitrarily set to 1 ($n = 3$; $^{**}P < 0.01$). **d** *Left panel* Western blot analysis for iNOS and CD16 in wild-type, *Col6a1*$^{-/-}$ and collagen VI-coated (5 μg/cm^2) *Col6a1*$^{-/-}$ BMDMs under control conditions or following induction with LPS. *Right panel* Densitometric quantification of iNOS vs. actin and CD16 vs. actin, as determined by three independent Western blot experiments. Values for the wild-type control group were arbitrarily set to 1 ($n = 3$–4; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). **e** Immunofluorescence for COX-2 in wild-type, *Col6a1*$^{-/-}$ and collagen VI-coated (5 μg/cm^2) *Col6a1*$^{-/-}$ BMDMs under control conditions or following induction with LPS. *Scale bar* 25 μm. *Col VI* collagen VI, *WT* wild type

compared to uninjured nerves (Fig. 6e). Finally, we normalized CD206 levels to CD68 levels and found that the relative amounts of CD206-positive M2 macrophages at 7 days post-injury were significantly lower in *Col6a1*$^{-/-}$ nerves than in wild-type nerves (Fig. 6f). Taken together, these data point to an impairment of macrophage M2 polarization in injured *Col6a1*$^{-/-}$ nerves.

To assess the in vivo role of AKT and PKA pathways in modulating collagen VI-mediated macrophage M2 polarization, we analyzed the activation of these two signals in injured wild-type and *Col6a1*$^{-/-}$ nerves. At 7 days post-crush, phosphorylation of both AKT and PKA was markedly enhanced in wild-type nerves, but not in *Col6a1*$^{-/-}$ nerves (Supplementary Fig. S7a, b). These findings support a role for the AKT and PKA pathways in the impaired macrophage M2 polarization and PNS regeneration of *Col6a1*$^{-/-}$ mice.

Collagen VI-regulated macrophage function contributes to PNS regeneration

To further confirm that the modulation of macrophage activities by collagen VI is crucial for PNS regeneration, we used macrophage-depleted in vivo models by injection of clodronate liposomes in wild-type and *Col6a1*$^{-/-}$ mice. Immunofluorescence for CD68 and F4/80 showed that injection of clodronate liposomes effectively depleted macrophages in sciatic nerves after crush (Supplementary Fig. S8). In the control group treated with PBS liposomes, the sciatic functional index was lower in *Col6a1*$^{-/-}$ mice than in wild-type mice, as expected (Fig. 7a). Following macrophage depletion by clodronate liposomes, the sciatic functional index was similarly reduced in wild-type and *Col6a1*$^{-/-}$ mice, thus indicating that the difference between the two genotypes was abolished after macrophage depletion (Fig. 7a). Moreover, the response to toe pinching was similar between wild-type and *Col6a1*$^{-/-}$ mice after macrophage depletion with clodronate liposomes, but was delayed when compared to control treatment with PBS liposomes (Fig. 7b). Similarly, analysis of the toe spreading reflex showed that depletion of macrophages significantly delayed the toe extension in both wild-type and *Col6a1*$^{-/-}$ mice, and it abolished the difference between the two genotypes (Supplementary Table S2). Toluidine blue staining of sciatic nerve cross sections showed that in mice treated with PBS liposomes the number of myelinated axons was significant higher in wild-type nerves than in *Col6a1*$^{-/-}$ nerves at 21 days post-injury, as expected (Fig. 7c). However, when mice were treated with clodronate liposomes, the difference in myelinated axon number between the two genotypes was completely abolished (Fig. 7c). These results indicate that defective macrophage recruitment is the main cause for the delayed PNS regeneration of collagen VI-deficient mice.

To directly investigate whether the delayed PNS regeneration of *Col6a1*$^{-/-}$ mice is due to the defects of collagen VI-regulated macrophage activities, we transplanted wild-type bone marrow cells into lethally irradiated wild-type mice (WT-WT) or collagen VI-deficient mice (WT-*Col6a1*$^{-/-}$). Functional studies showed that there were no significant differences between WT-WT and WT-*Col6a1*$^{-/-}$ mice in the sciatic functional index score (Fig. 8a), time to initial response to toe pinch in digits 3, 4 and 5 (Fig. 8b) and time to initial toe extension (Fig. 8c), indicating that the delayed PNS regeneration in *Col6a1*$^{-/-}$ mice is rescued by transplantation of wild-type bone marrow cells. Next, we investigated whether the transplanted wild-type cells were able to rescue the decreased macrophage recruitment and polarization of *Col6a1*$^{-/-}$ mice after nerve crush injury. Immunofluorescence for CD68 and F4/80 showed comparable CD68- and F4/80-positive macrophages in sciatic nerves of WT-WT and WT-*Col6a1*$^{-/-}$ mice at 7 days post-injury (Fig. 8d). Western blot analysis showed that CD206 levels were similar between WT-WT and WT-*Col6a1*$^{-/-}$ mice at 7 days post-injury (Fig. 8e). Taken together, these findings provide evidence demonstrating that the delayed PNS regeneration in *Col6a1*$^{-/-}$ mice is induced by the deficits in macrophage migration and polarization.

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | ☐ LE | ☐ TYPESET
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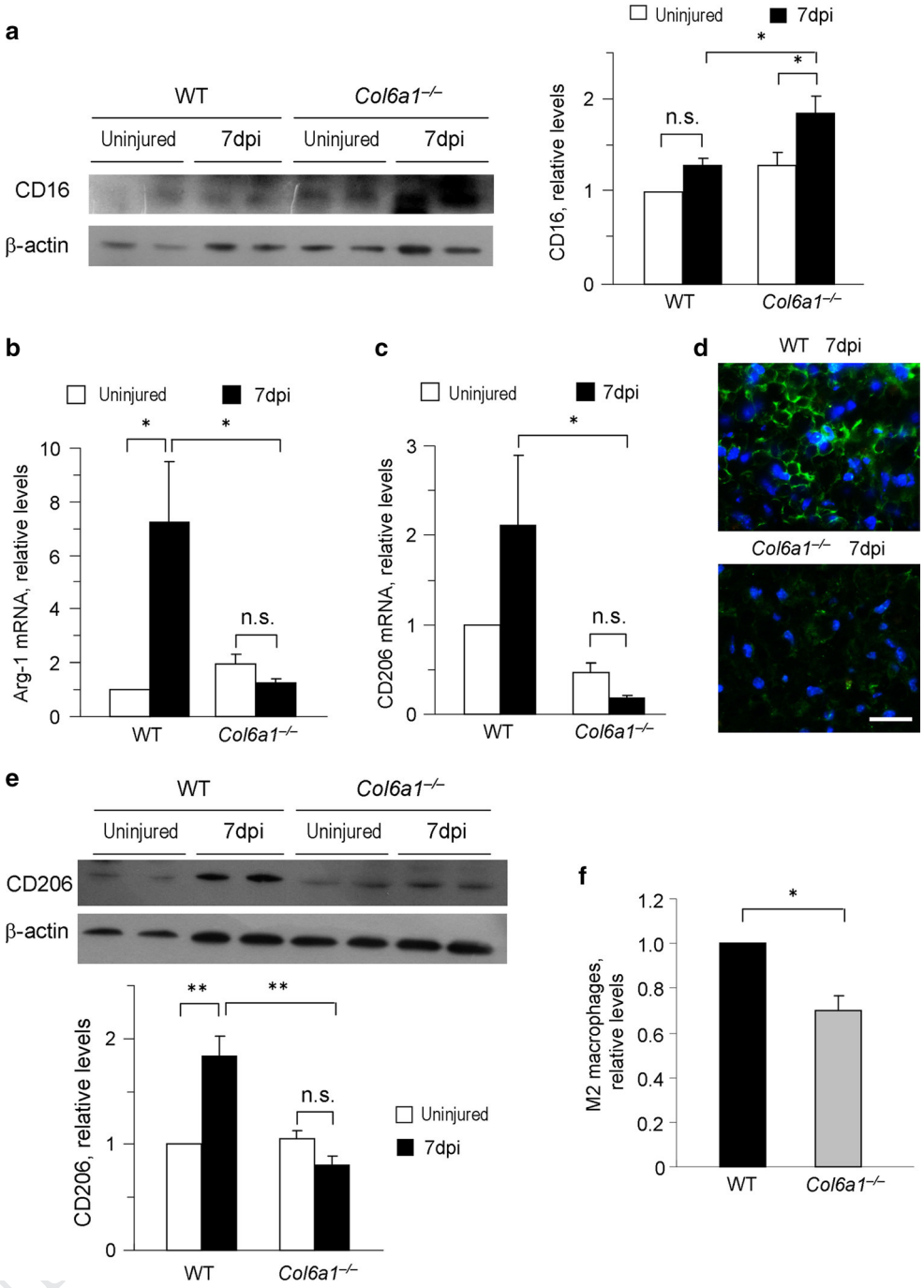

Fig. 6 Ablation of collagen VI decreases macrophage M2 polarization after nerve injury. **a** *Left panel* Western blot analysis for CD16 in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. *Right panel* Densitometric quantification of CD16 vs. actin, as determined by three independent Western blot experiments. Values for uninjured wild-type nerves were arbitrarily set to 1 ($n = 4$; $^{*}P < 0.05$; *n.s.* not significant). Real-time RT-PCR analysis for Arg-1 (**b**) and CD206 (**c**) mRNA in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. Values for uninjured wild-type nerves were arbitrarily set to 1. GAPDH was used as a reference gene ($n = 3$–5; $^{*}P < 0.05$; *n.s.* not significant). **d** Immunofluorescence for CD206 in cross sections of sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice at 7 days post-crush. *Scale bar* 25 μm. **e** *Top panel* Western blot analysis for CD206 in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice under uninjured conditions and at 7 days post-crush. *Bottom panel* Densitometric quantification of CD206 vs. actin as determined by three independent Western blot experiments. Values for uninjured wild-type nerves were arbitrarily set to 1 ($n = 4$; $^{**}P < 0.01$; *n.s.* not significant). **f** Quantification of CD206 vs. CD68 in sciatic nerves from wild-type and *Col6a1*$^{-/-}$ mice at 7 days post-crush as determined by three independent Western blot experiments. Values for uninjured wild-type nerves were arbitrarily set to 1 ($n = 4$; $^{**}P < 0.05$). *dpi* days post-injury, *WT* wild type

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | LE | TYPESET
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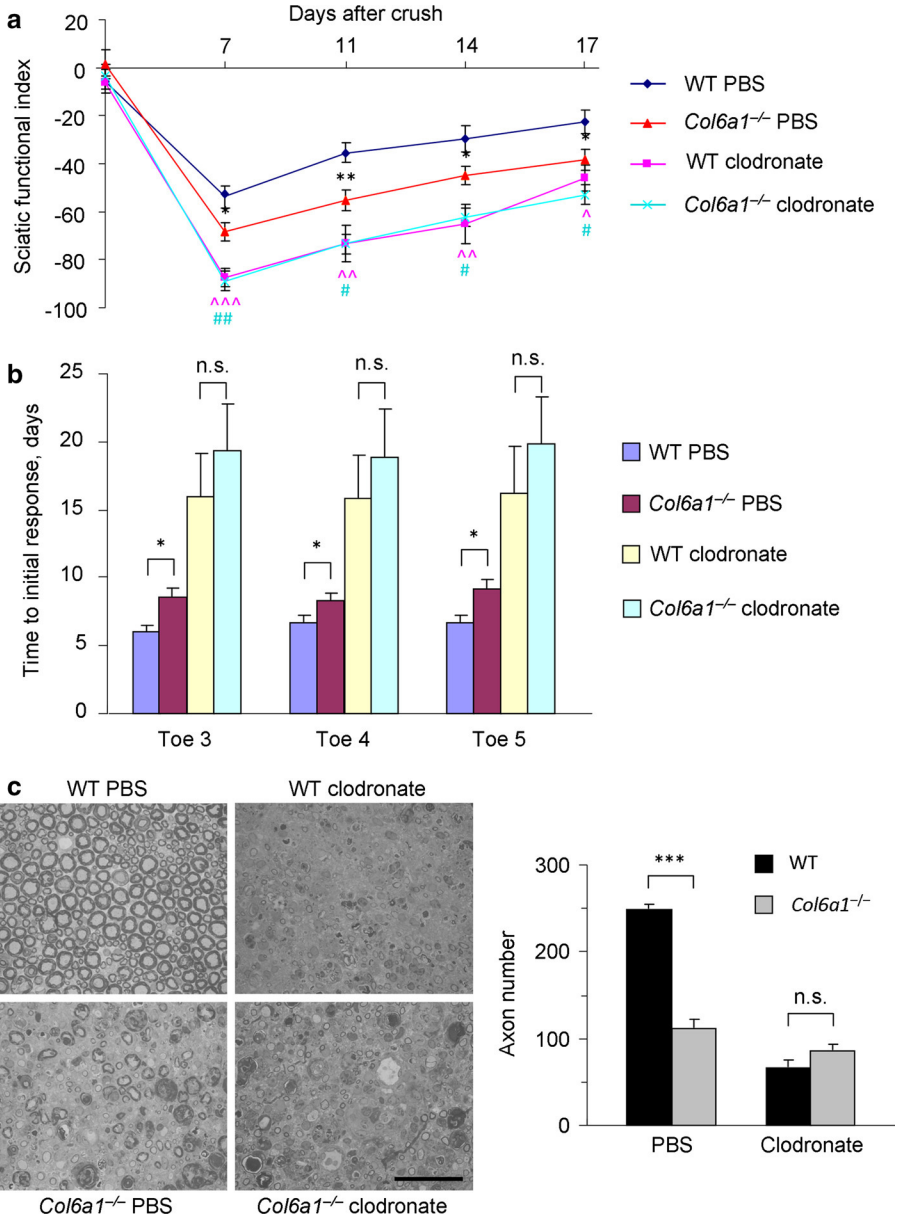

Fig. 7 Macrophage depletion leads to similar regenerative responses in wild-type and collagen VI-deficient peripheral nerves. **a** Quantification of the sensory-motor function of wild-type and *Col6a1*$^{-/-}$ mice under control conditions (PBS liposomes) and after macrophage depletion (clodronate liposomes) by analyzing the sciatic functional index from footprint tracks before crush and at 7, 11, 14 and 17 days post-crush ($n = 5$–7; $^{*}P < 0.05$ and $^{**}P < 0.01$, *Col6a1*$^{-/-}$ PBS vs. wild-type PBS; $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$ and $^{\wedge\wedge\wedge}P < 0.001$, wild-type clodronate vs. wild-type PBS; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, *Col6a1*$^{-/-}$ clodronate vs. *Col6a1*$^{-/-}$ PBS). **b** Quantification of the sensory function of wild-type and *Col6a1*$^{-/-}$ mice under control conditions (PBS liposomes) and after macrophage depletion (clodronate liposomes) by recording the initial response time to the pinch using forceps in the digits 3, 4 and 5 after sciatic nerve crush ($n = 5$–7; $^{*}P < 0.05$; *n.s.* not significant). **c** Representative images of toluidine blue staining and morphometric analysis of the myelinated axon number in cross sections of injured sciatic nerves at 21 days post-crush from wild-type and *Col6a1*$^{-/-}$ mice that received PBS liposomes or clodronate liposomes. *Scale bar* 40 μm ($n = 3$; $^{***}P < 0.001$; *n.s.* not significant). *dpi* days post-injury, *WT* wild type

Discussion

Our previous work demonstrated that collagen VI is required for muscle regeneration [46], suggesting a role in tissue repair. The results presented in this study show that collagen VI promotes PNS regeneration by regulating macrophage recruitment and polarization. Lack of collagen VI in *Col6a1*$^{-/-}$ mice prevents macrophage recruitment and phenotypic transition after sciatic nerve crush, which in turn inhibits PNS regeneration. Previous studies showed that macrophages play a pivotal function in Wallerian degeneration by clearing myelin debris and in axonal regeneration by secreting a variety of soluble factors [33]. However, the molecular mechanisms underlying macrophage recruitment into injured nerves are not well understood. It is well accepted that soluble factors secreted by the disrupted axon/Schwann cell nerve unit are responsible for macrophage recruitment following nerve injury [33, 45]. Among these factors, IL-1β and MCP-1 are two major macrophage chemoattractants in injured peripheral nerves [36, 41, 45]. However, blockade of IL-1β and MCP-1 with function-blocking antibodies does not completely inhibit macrophage recruitment into injured peripheral nerves in vivo [36]. Similar effects were also displayed by in vitro experiments, where addition of MCP-1 neutralizing antibodies to conditioned media from Schwann cell cultures and nerve segments does not completely block macrophage migration [45]. These findings indicate that other chemoattractants are also secreted by the injured peripheral nerves. In the current study, we found that collagen VI promotes

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | LE | TYPESET
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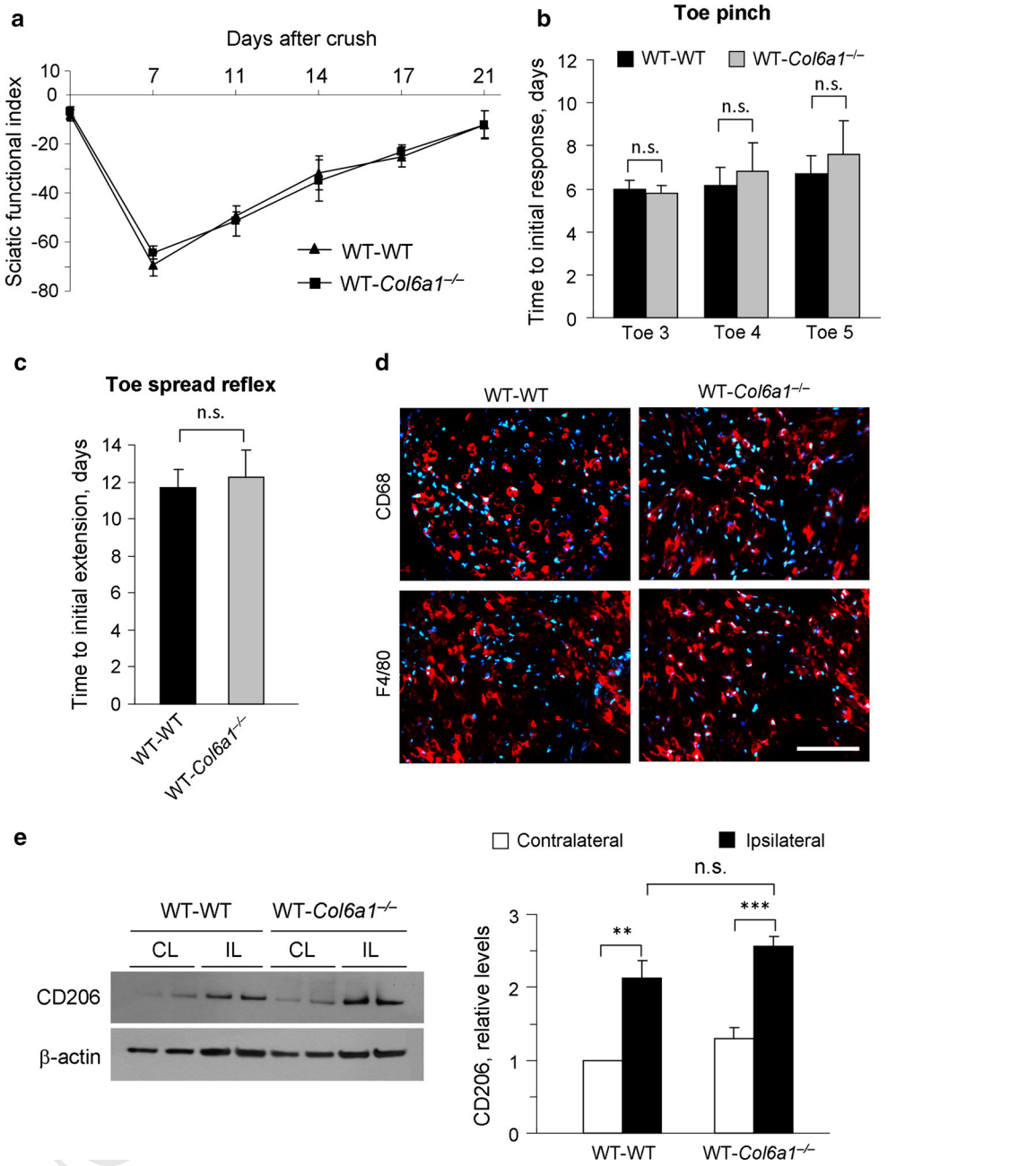

Fig. 8 Transplantation of wild-type bone marrow cells into *Col6a1*$^{-/-}$ host mice rejuvenates regeneration and macrophage activities after nerve injury. **a** Quantification of sensory-motor function of wild-type bone marrow cells transplanted into wild-type mice (WT-WT) and of wild-type bone marrow cells transplanted into *Col6a1*$^{-/-}$ mice (WT-*Col6a1*$^{-/-}$) by analyzing the sciatic functional index from footprint tracks before crush and at 7, 11, 14, 17 and 21 days post-crush (n = 5–7). **b** Quantification of sensory function of WT-WT and WT-*Col6a1*$^{-/-}$ mice after sciatic nerve crush by recording the initial response time (day post-injury) to the pinch using forceps in the digits 3, 4 and 5 (n = 5–7; *n.s.* not significant). **c** Quantification of motor function of WT-WT and WT-*Col6a1*$^{-/-}$ mice after sciatic nerve crush by recording the initial extension time (day post-injury) to the toe spreading reflex (n = 4–6; *n.s.* not significant). **d** Immunofluorescence for CD68 and F4/80 in cross sections of injured sciatic nerves from WT-WT and WT-*Col6a1*$^{-/-}$ mice at 7 days post-crush. *Scale bar* 50 μm. **e** *Left panel* Western blot analysis for CD206 in sciatic nerves from WT-WT and WT-*Col6a1*$^{-/-}$ mice at 7 days post-crush. *Right panel* Densitometric quantification of CD206 vs. actin as determined by three independent Western blot experiments. Values for uninjured WT-WT contralateral nerves were arbitrarily set to 1 (n = 4; $^{**}P < 0.01$; $^{***}P < 0.001$; *n.s.* not significant). *CL* contralateral, *IL* ipsilateral, *WT-Col6a1*$^{-/-}$ wild-type bone marrow cells transplanted into *Col6a1*$^{-/-}$ mice, *WT-WT* wild-type bone marrow cells transplanted into wild-type mice

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | LE | TYPESET
MS Code : ANEU-D-14-00635 | CP | DISK

macrophage migration both in vitro and in vivo and that *Col6a1*$^{-/-}$ macrophages exhibit a reduced migration capability in the Matrigel plug assay. In the sciatic nerve crush model, macrophage recruitment was markedly impaired in *Col6a1*$^{-/-}$ nerves. Thus, we identified collagen VI as a novel chemoattractant that triggers macrophage recruitment into injured nerves.

Our recent work demonstrated that in peripheral nerves collagen VI is abundantly deposited by Schwann cells and macrophages, but not by axons [7]. Upon injury in the PNS, Schwann cells dedifferentiate to a progenitor/stem cell-like state [35], expressing high levels of collagen VI [48]. In this study, we found that the expression of collagen VI is significantly upregulated upon sciatic nerve injury. On the one hand, this enhancement is likely contributed by the dedifferentiated Schwann cells; on the other hand, it is related to the increased number of macrophages after injury. In this regard, it is plausible that at the initial stage the dedifferentiated Schwann cells are responsible for increasing collagen VI deposition in injured nerves, which in turn promotes macrophage recruitment in a paracrine manner. Thereafter, both paracrine and autocrine effects may exist for the collagen VI contribution to macrophage recruitment. Our findings demonstrate that the impaired macrophage recruitment in injured *Col6a1*$^{-/-}$ nerves is rescued by transplantation of wild-type bone marrow cells, highlighting the autocrine effect of collagen VI in macrophage recruitment after peripheral nerve injury. Our data indicate that collagen VI acts as a chemoattractant for macrophages, a finding that is fully consistent with previous studies showing that certain ECM proteins, such as fibronectin, laminin and collagen IV, exhibit specific chemoattractant activities for different cells [1, 24, 49]. In addition to directly exhibiting chemotactic activity, collagen VI also influence the expression of other chemoattractants. For example, we found in this study that the upregulation of IL-1β and MCP-1 induced by sciatic nerve crush injury is significantly impaired in *Col6a1*$^{-/-}$ mice, suggesting that collagen VI is able to promote the recruitment of macrophages into the injured nerves through a variety of molecular mechanisms.

The function of macrophages in PNS regeneration is also related to their phenotype, where M2 macrophages stimulate regeneration [30]. Therefore, macrophage polarization from the M1 to M2 phenotype is crucial for successful PNS regeneration. It has been shown that acute peripheral nerve injury elicits an M2 macrophage response [50]. However, the mechanisms that trigger and modulate macrophage polarization are not well understood. To date, it is known that macrophage polarization is largely controlled by a small group of signals and factors, such as nuclear factor κB (NF-κB), mammalian target of rapamycin (mTOR), signal transducer and activator of transcription 6 (STAT6), PPARγ, Kruppel-like factor 4 (KLF4), AKT and PKA [4, 25, 27, 38]. In this study, we identified collagen VI as a novel factor regulating macrophage polarization. We found that addition of collagen VI promotes the J774 macrophage polarized toward the M2 phenotype. In the light of these findings, we isolated primary BMDMs and PMs from wild-type and *Col6a1*$^{-/-}$ mice and stimulated their polarization into M1 and M2 phenotypes with LPS and IL-4, respectively. Consistent with our hypothesis, deficiency of collagen VI impairs macrophage M2 polarization and promotes macrophage M1 polarization, which can be reversed by addition of purified native collagen VI.

Our findings indicate that collagen VI plays a pivotal role in macrophage polarization. Although one recent in vitro work showed that some specific ECM components, namely collagen I and fibronectin, are not needed for macrophage polarization [37], previous evidence suggests that ECM plays a key role in this process. For example, ECM-derived biologic scaffolds induce an in vivo constructive tissue remodeling by promoting an M2 macrophage response [42]. In a myocardial infarction model, deficiency of matrix metalloproteinase 28 (MMP-28) was found to attenuate macrophage M2 polarization and reduce the expression of several ECM genes [28]. Among the different ECM molecules, collagen VI seemed to be the best candidate for regulating macrophage polarization. M2 macrophages produce higher levels of collagen VI than M1 macrophages [40]. Moreover, macrophages in the adipose tissue of insulin-resistant subjects are associated with collagen VI deposition and exhibit M2 phenotype [44]. These indirect observations prompted us to investigate the role of this ECM molecule for macrophage polarization. In addition to in vitro data, we obtained direct in vivo evidence showing that collagen VI regulates macrophage polarization during PNS regeneration. We found that at 7 days post-injury, the levels of M2 macrophages were decreased in *Col6a1*$^{-/-}$ nerves, indicating an impairment of macrophage skewing, which in turn inhibits PNS regeneration.

Notably, the different nerve regeneration response of wild-type and *Col6a1*$^{-/-}$ mice is abolished by in vivo macrophage depletion. Moreover, our data demonstrate that the defective PNS regeneration of *Col6a1*$^{-/-}$ mice, and the defective injury-induced macrophage migration and polarization, are rescued by transplantation of wild-type bone marrow cells. Together, these findings highlight the mechanistic insight of collagen VI regulation of macrophage activities as a critical player for PNS regeneration. In addition, we provide evidence showing that the AKT and PKA pathways contribute to collagen VI-regulated macrophage function. Addition of collagen VI to cultured macrophages promotes the activation of AKT and PKA, whereas collagen VI ablation abolishes IL-4-induced activation of both signals. Blockade of AKT and PKA by their inhibitors abrogates collagen VI-induced

Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | ☐ LE | ☐ TYPESET
MS Code : ANEU-D-14-00635 | ☑ CP | ☑ DISK

macrophage migration and polarization. Furthermore, our in vivo data indicate that the increased activation of AKT and PKA in injured nerves is completely inhibited by collagen VI ablation. Our results on the one hand support the concept that AKT and PKA pathways are necessary for macrophage migration and polarization [3, 10, 11, 27, 38] and axonal regeneration [9, 17]; on the other hand, they provide insights into the downstream targets of collagen VI-regulated macrophage function in PNS regeneration.

In summary, we demonstrate in this study that collagen VI is a pivotal factor for macrophage function. In this context, besides providing novel molecular understanding for macrophage migration and polarization, our study points out potentially broad implications for collagen VI in inflammatory diseases. These data also provide evidence for a beneficial impact of collagen VI on peripheral nerve regeneration via modulation of macrophage activities. In addition to contributing to the understanding of the roles of collagen VI in the experimental setting of PNS regeneration, our findings might have useful implications for clinical study. For example, it is reasonable that application of collagen VI as a coating substrate for the artificial nerve guide conduits may be beneficial for improving the peripheral nerve functional recovery in patients. Future studies will allow testing this possibility and evaluating the effectiveness of such regenerative approaches.

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Conflict of interest The authors declare no potential conflicts of interest.

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Journal : Large 401 | Dispatch : 21-11-2014 | Pages : 17
Article No : 1369 | ☐ LE | ☐ TYPESET
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Article No : 1369 | ☐ LE | ☐ TYPESET
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Journal:	**401**
Article:	**1369**

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