Research Article

Bioinformatic analysis of cytokine expression in the proximal and distal nerve stumps after peripheral nerve injury

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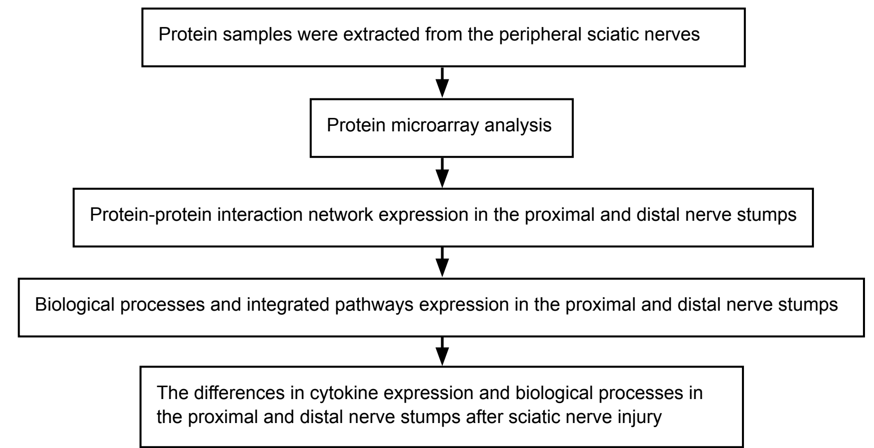
Graphical Abstract *The difference of the precise mechanisms in cytokine expression between the proximal and distal nerve stumps after peripheral nerve injury*



Abstract

In our previous study, we investigated the dynamic expression of cytokines in the distal nerve stumps after peripheral nerve injury using microarray analysis, which can characterize the dynamic expression of proteins. In the present study, we used a rat model of right sciatic nerve transection to examine changes in the expression of cytokines at 1, 7, 14 and 28 days after injury using protein microarray analysis. Interleukins were increased in the distal nerve stumps at 1–14 days post nerve transection. However, growth factors and growth factor-related proteins were mainly upregulated in the proximal nerve stumps. The *P*-values of the inflammatory response, apoptotic response and cell-cell adhesion in the distal stumps were higher than those in the proximal nerve stumps, but the opposite was observed for angiogenesis. The number of cytokines related to axons in the distal stumps was greater than that in the proximal stumps, while the percentage of cytokines related to axons in the distal stumps was lower than that in the proximal nerve stumps. Visualization of the results revealed the specific expression patterns and differences in cytokines in and between the proximal and distal nerve stumps. Our findings offer potential therapeutic targets and should help advance the development of clinical treatments for peripheral nerve injury. Approval for animal use in this study was obtained from the Animal Ethics Committee of the Chinese PLA General Hospital on September 7, 2016 (approval No. 2016-x9-07).

Key Words: cytokine; distal stumps; Gene Ontology; Kyoto Encyclopedia of Genes and Genomes Pathway; microarray; microenvironment; peripheral nerve injury; proximal stumps

Chinese Library Classification No. R446; R741; R318.04

Introduction

Peripheral nerve injury (PNI) is often caused by traffic accidents, trauma or surgery, resulting in pain and the loss of nerve function (Luo et al., 2020; Yuan et al., 2020). In contrast to the central nervous system (CNS), nerves in the peripheral nervous system have an inherent ability to regenerate. However, clinical therapy cannot completely restore the functional connections of nerves (Gruart et al., 2003). This is because the regeneration and functional connection of peripheral nerves involves a complex process that consists of macrophage invasion, axon degeneration and Schwann cell proliferation, as well as requiring a permissive microenvironment for axon growth (Zochodne, 2000; Parrinello et al., 2010; Rishal and Fainzilber, 2010).

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Wallerian degeneration (WD) is common during peripheral nerve degeneration. As for spinal cord injury (Stoll et al., 2002), many studies have explored the cellular response and molecular mechanisms of WD after PNI (Rotshenker, 2011; Tricaud and Park, 2017; Wei et al., 2020). Soon after injury, a series of immune and inflammatory responses occur: the number of macrophages sharply increases, and they are recruited to the injury region; Schwann cells start to demyelinate and secrete numerous cytokines and growth factors; and these cells together digest myelin fragments shed by demyelinating Schwann cells (Yi et al., 2015). Many studies on nerve regeneration have focused on the microenvironment in the distal nerve stump (Wang et al., 2017; Xing et al., 2017). Yi et al. (2015) examined the differential expression of microRNAs at various time points after PNI. Subsequently, Yu et al. (2016) examined the expression patterns of different genes during WD.

Notably, axons of the distal stumps inevitably lose contact with the neuronal cell body during WD after PNI (Zhang et al., 2010), while axons remain in contact with the cell bodies in the proximal stumps of injured nerves. Axon regeneration from the proximal stumps to the distal stumps is the reconstructive event necessary for peripheral nerve regeneration. This suggests that the microenvironment of the proximal stumps might also be important. However, to date, less attention has been given to the proximal nerve stumps in regeneration research. Furthermore, the differences in the microenvironment between the proximal and distal stumps after PNI remain unknown.

In our previous study, we investigated the dynamic expression of cytokines in the distal nerve stumps after PNI using microarray analysis, which can characterize the dynamic changes in proteins (Cheng et al., 2020). In the present study, we first examined the spatiotemporal changes in cytokines in the proximal stump of the sciatic nerve, and then focused on the differences between the proximal and distal stumps. Furthermore, we used bioinformatics analysis to decipher the related signaling pathways and biological processes, helping to further our understanding of peripheral nerve regeneration.

Materials and Methods

Animal preparation and surgery

A total of 60 specific pathogen-free male Sprague-Dawley rats, 8 weeks of age and weighing 200–250 g, were provided by the Experimental Animal Research Center at the Chinese PLA General Hospital (license No. SCXK (Jing) 2016-002). The experiments were approved by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital (approval No. 2016-x9-07) on September 7, 2016. All animals were kept in a clean environment with appropriate temperature (20–22°C) and humidity conditions (50–60%).

The animals were randomly divided into the following five groups (12 rats in each group): control, 1 day postoperative, 7 days postoperative, 14 days postoperative, and 28 days postoperative. All rats were anesthetized via intraperitoneal injection of 3% (w/v) sodium pentobarbital solution (2.5 mg/100 g body weight) and underwent surgical transection of sciatic nerves as previously described (Cheng et al., 2020). Briefly, the sciatic nerve was exposed at the midthigh level of the right hind limb and transected. The rats were sacrificed at 1, 7, 14 or 28 days after nerve transection. The rats in the control group underwent sham surgery of the right sciatic nerve. The proximal nerve stumps were dissected and stored at –80°C until use.

Protein microarray and bioinformatics analysis

Protein samples were extracted from the proximal nerve stumps of sciatic nerves using lysis buffer containing a protease inhibitor cocktail (Pulilai, Beijing, China). Microarray analysis was performed using the Rat Cytokine Array 67 (RayBiotech, Guangzhou, China), as described previously (Cheng et al., 2020). The microarray signals were visualized using the LuxScan 10K scanner (CapitaBio, Beijing, China) at a 532 nm wavelength. Proteins with an expression fold change > 2 or < 0.5 and adjusted *P*-value < 0.05 were considered significantly differentially expressed. The selected proteins were transformed into corresponding genes and mapped using GeneMANIA (http://genemania.org) to evaluate the interactive relationships among them. Then, protein-protein interaction (PPI) networks were constructed using Cytoscape software (http://www.cytoscape.org). Both Gene Ontology (GO) analysis (http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (http://www.genome.jp/kegg/) are useful methods for identifying biological processes and canonical pathways. Bioinformatic analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://www.david.niaid.nih.gov) online tool.

Metascape analysis

Metascape analysis (http://metascape.org) was performed to analyze the cytokine overlaps and biological process differences between the proximal stumps and distal stumps (Zhou et al., 2019). Metascape supports gene identifier types, such as Entrez Gene ID, Ensembl ID, RefSeq and UniProt ID. Gene (corresponding to its cytokine) lists were tested by custom analysis. Circos plots were used to visualize the overlaps among multiple lists (e.g., distal and proximal). Heatmaps were used to show the top enrichment clusters (e.g., GO biological process, KEGG pathways) using a discrete color scale to indicate statistical significance and gray color to indicate nonsignificance. Pie charts were used to visualize the enrichment of membership search terms related to "axon".

Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM), and analyzed with GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Differences between groups were tested using Student's *t*-test or one-way analysis of variance.

Results

Overview of cytokine changes in the proximal nerve stumps at 1, 7, 14 and 28 days post nerve transection

In our previous study, we investigated the spatiotemporal changes in cytokines in the distal nerve stump after sciatic nerve injury (Cheng et al., 2020). In the present study, we investigated the differences in protein expression between the distal and proximal sciatic nerve stumps. We began by first examining cytokine expression in the proximal stump with the Rat Cytokine Array 67. The assay was performed on sciatic nerves sampled from each group (n = 4 each) at 1, 7, 14 and 28 days post nerve transection. Principal component analysis was used to assess the cytokine expression in these completely independent groups (**Figure 1F**). The resulting differentially expressed cytokines are illustrated by heatmaps (three replicates per group) (**Figure 1A**–**D**). A Venn diagram was used to compare the differentially expressed cytokines among the various groups (**Figure 1E**). Following injury, a comparable number of cytokines changed expression at different time points. Galectin-1, Galectin-3, glial cell-derived neurotrophic factor family receptor alpha-1 (GFR alpha-1), monocyte chemoattractant protein-1 (MCP-1) and Neuropilin-1 were upregulated at all time points, while nerve growth factor beta was downregulated. Some cytokines showed dramatic changes in protein expression at several time points. Thymus chemokine-1 and metallopeptidase inhibitor 1 increased during early injury (at 1–7 days); Gas 1, intercellular adhesion molecule 1 and macrophage inflammatory protein-1a increased during the middle period (at 7–14 days); and

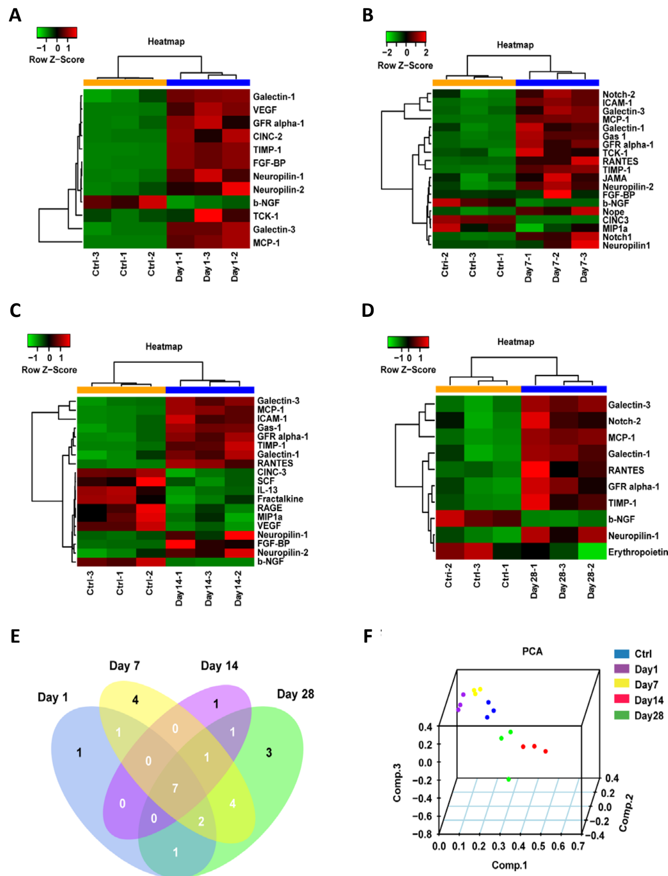

Figure 1 | Schematic diagram of differentially expressed cytokines in the proximal nerve stumps following sciatic nerve transection.
(A–D) Heatmap and hierarchical clustering of the different cytokines. (A) Control and 1 day postoperative groups. (B) Control and 7 days postoperative groups. (C) Control and 14 days postoperative groups. (D) Control and 28 day postoperative groups. Red indicates upregulated cytokines compared with the control group (fold change > 2), while green indicates downregulated cytokines (fold change ≤ 0.5). (E) Venn diagram of the differentially expressed cytokines at different time points following sciatic nerve transection. (F) Principal component analysis of the five groups at different time points following sciatic nerve transection. Comp.: Principal component.

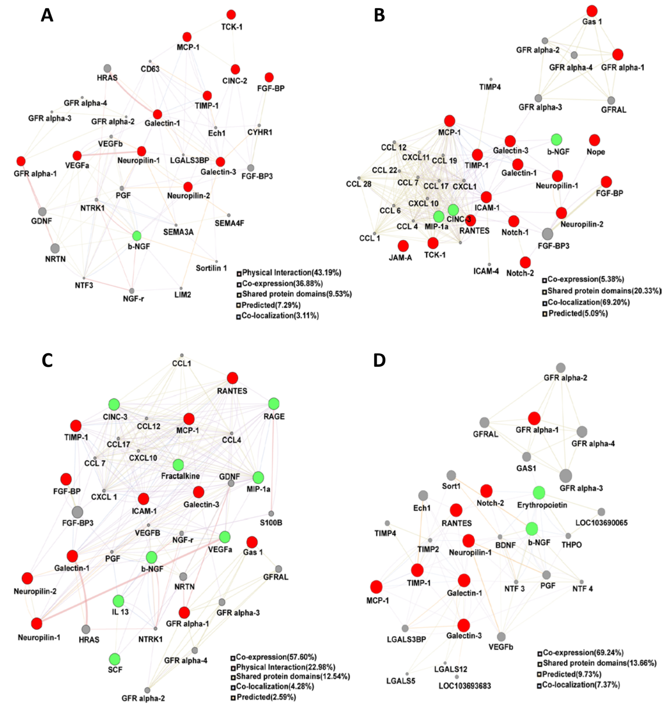

Figure 2 | Protein-protein interaction networks based on the upregulated and downregulated cytokines in the proximal stumps (enrichment analysis using GeneMANIA).
(A–D) Network of identified targets at 1 (A), 7 (B), 14 (C) and 28 (D) days after sciatic nerve transection. The sizes of the spots in the network represent the weights of the cytokines/proteins in the network. The red spots represent the upregulated cytokines, and the green spots represent the downregulated cytokines. The gray spots represent the selected proteins potentially related to the differentially expressed cytokines. Enrichment analysis was performed with GeneMANIA, a Cytoscape plugin (http://genemania.org).

metallopeptidase inhibitor 1 increased during the late period (at 14–28 days). Furthermore, the protein expression levels of cytokine-induced neutrophil chemoattractant 2, fibroblast growth factor-binding protein (FGF-BP) and neuropilin-2 were increased at 1–14 days post nerve transection. Regulated upon activation, normal T cell expressed and secreted (RANTES) was expressed at higher levels at 7–28 days post nerve transection. In addition, some cytokines were upregulated at only one time point. For example, junctional adhesion molecule (JAM)-A, Nope and Notch-1 increased at 7 days post nerve transection; Fractalkine, interleukin (IL)-13, receptor for advanced glycation end products, and stem cell factor increased at 14 days post nerve transection; and erythropoietin increased at 28 days post nerve transection.

PPI networks of the differentially expressed proteins following sciatic nerve injury

The differentially expressed cytokines obtained from the protein microarray were analyzed in GeneMANIA, a Cytoscape plugin, to construct PPI networks. The plugin generated a complex network comprising the top 20 nodes (interacting proteins) and five types of edges (interactions), which are shown in **Figure 2**.

At 1 day post nerve transection, Cd63, GFR alpha-2, GFR 3, GFR 4, secreted semaphorin (Sema) 3a and Sema4f, among others, were found to interact with the differentially expressed cytokines. Of note, Sema3a plays key roles in axonal guidance and neural regeneration. Inhibitors of Sema3a-induced chemorepulsion promote the neural regeneration of damaged axons (Montolio et al., 2009). Sema3a induces growth cone collapse, thereby inhibiting regeneration after rat sciatic nerve crush (Yao et al., 2016). At 7 days post nerve transection, more chemokines (previously identified in the positive regulation of chemotaxis and transport to mediate immune and inflammatory responses) were involved, including CC motif chemokine ligand (CCL)1, CCL4, CCL7, CCL12, CCL6, CCL17, CCL22, CCL28, C-X-C motif chemokine ligand (CXCL)1, CXCL9, CXCL10 and CXCL11. CCL4 (also known as macrophage inflammatory protein-1β) is increased in macrophages and Schwann cells, and regulates neuropathic pain by stimulating inflammatory mediator secretion after sciatic nerve injury (Saika et al., 2012). CXCL9, CXCL10 and CXCL11 are also closely related to neuroinflammation and neurodegeneration (Koper et al., 2018). At 14 days post nerve transection, in addition to some chemokines, other differential proteins were identified, including S100b, vascular endothelial growth factor (VEGF)b, placental growth factor, FGF-BP3, neurotrophic tyrosine kinase receptor type 1, glial cell-derived neurotrophic factor, Gfra, Gfra3 and Gfra4. S100b, a Schwann cell marker, is essential for the development of Schwann cells. VEGFB, glial cell-derived neurotrophic factor and nerve growth factor receptor play crucial roles in neuronal survival, differentiation and growth. At 28 days post nerve transection, the interacting proteins included placental growth factor, brain-derived neurotrophic factor (BDNF), VEGFb, tissue inhibitors of metalloproteinases (Timp)2 and Timp4, Sort1, Ntf3, Ntf4, Gas1, Gfra2, Gfra3, Gfra4, Lgals12 and Lgals3bp, instead of chemokines. A neurotrophin, BDNF is essential for neuronal survival and function. Elevated BDNF expression prevents neuronal death, accelerates neuronal activity, and promotes axonal regeneration (Henderson et al., 1993; Braun et al., 1996; Lykissas et al., 2007).

Biological processes and integrated pathways following sciatic nerve injury

To identify critical signaling pathways, KEGG analysis was performed at different time points. Cytokine–cytokine receptor interaction, a typical KEGG pathway, was activated at all time points from 1 to 28 days post nerve transection. Furthermore, the chemokine signaling pathway was significantly involved at 1, 7 and 14 days post nerve transection. At 1, 14 and 28 days post nerve transection, the Rap1, Ras and phosphoinositide 3-kinase-Akt signaling pathways were significantly enriched. The nucleotide oligomerization domain-like receptor signaling pathway was observed at only 7 days post nerve transection. At 7 and 14 days post nerve transection, two KEGG pathways, the Toll-like receptor signaling pathway and Influenza A, were activated. In the later stage (at 28 days post nerve transection), the Janus kinase-signal transducer and activator of transcription (Jak–STAT) signaling pathway and mitogen-activated protein kinase signaling pathway were observed. In addition, some diseases were significantly enriched following nerve injury. For example, rheumatoid arthritis was related to the response to nerve injury at 1, 7 and 14 days post nerve transection. Chagas disease (American trypanosomiasis) and malaria were enriched at 7 days post nerve transection (**Figure 3** and **Additional Table 1**).

To further elucidate the biological processes involved in the spatiotemporal changes in cytokines, the DAVID database was used to identify GO terms at various time points post-sciatic nerve transection. The top 10 enriched categories of biological processes are listed, with *P*-values less than 0.05. In brief, at 1 day post nerve transection, axon extension, axon guidance, axonogenesis, sympathetic ganglion development, sympathetic neuron projection guidance and extension were significantly activated. At 7–14 days post nerve transection, other canonical biological processes emerged, including cell chemotaxis and immune and inflammatory responses. At 28 days post nerve transection, neuron apoptotic process, cell proliferation, and the response to lipopolysaccharide were involved (**Figure 4** and **Additional Table 2**).

Differences in cytokine expression in the proximal and distal stumps after sciatic nerve injury

To provide insight into the differences in the microenvironments in the proximal and distal stumps after sciatic nerve injury, we first analyzed differences in the expression of upregulated cytokines at different time points.

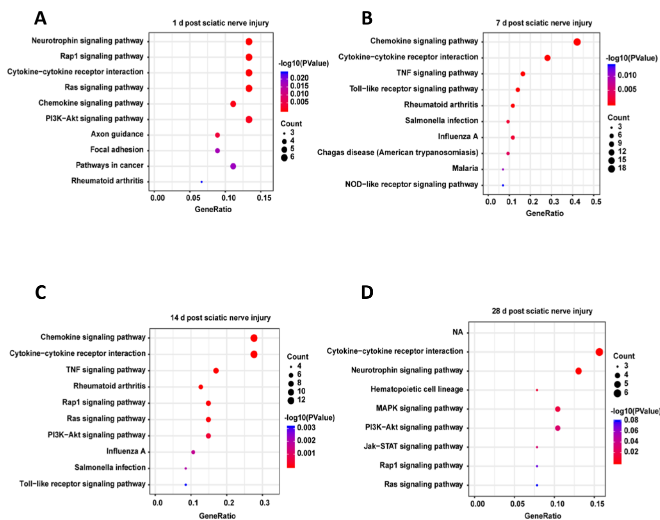

Figure 3 | KEGG pathways enriched among the proteins in the proximal stumps in the protein-protein interaction network.
The *X*-axis shows the gene ratio, which is the ratio of the specific cytokine numbers to all numbers annotated in the KEGG pathway. The *Y*-axis shows the top ten KEGG pathways with $P < 0.05$. KEGG: Kyoto Encyclopedia of Genes and Genomes.

Interestingly, the cytokines in the proximal and distal nerve stumps showed similar and distinct changes at each time point; the results are shown in a circos plot (**Figure 5**). The number of similarly changed cytokines displayed a curved shape in both the proximal and distal stumps in the injured sciatic nerve. Interestingly, there were some differences between these two injured regions. For example, at 1–14 days post nerve transection, interleukins were primarily increased in the distal stumps—IL-4, IL-1 R6, IL-10 and gp130 (IL6ST) were significantly upregulated at 1 day after nerve transection. In addition to these interleukins, IL-7 and IL-1ra were also

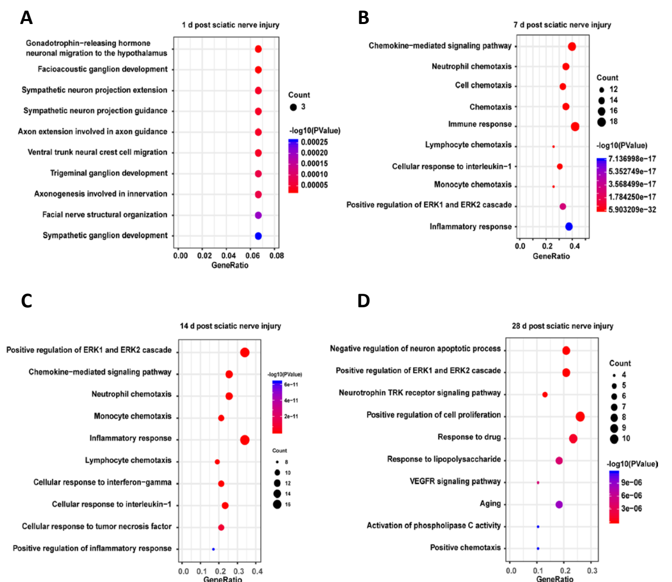

Figure 4 | GO biological processes enriched among the proteins in the proximal stumps in the protein–protein interaction network.
The *X*-axis shows the gene ratio, which is the ratio of the specific cytokine numbers to all numbers annotated in the GO pathway. The *Y*-axis shows the top ten GO pathways with $P < 0.05$. GO: Gene Ontology.

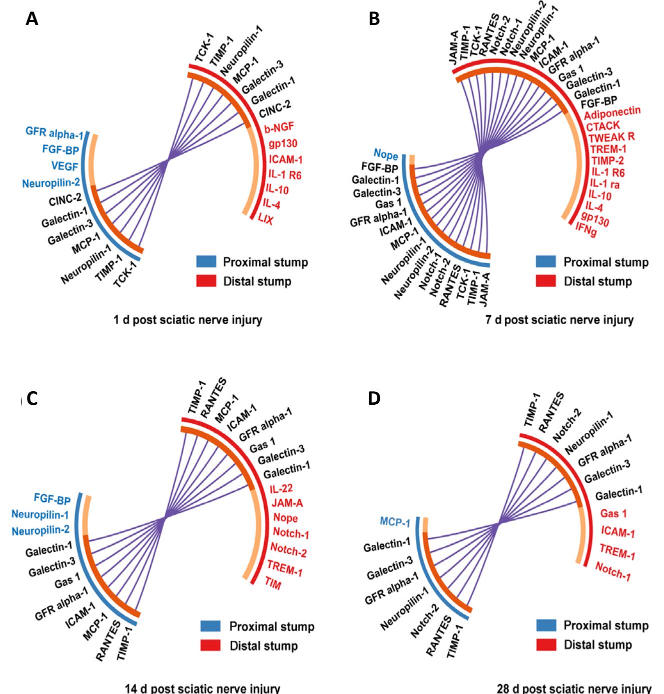

Figure 5 | Circos plot visualization of the overlaps among the cytokine lists of the proximal stump and distal stump analyzed with Metascape.
The blue color represents the proximal stump, and the red color represents the distal stump (each arc on the outside). The dark orange color represents the same cytokines in the two lists, and the light orange color represents the unique cytokines in each of the two lists (each arc on the inside).

markedly increased at 7 days post nerve transection. Only IL-22 had a high level of protein expression. There were fewer upregulated cytokines in the distal nerve stumps than in the proximal nerve stumps—VEGF, growth factor-related proteins (FGF-BP, GFR alpha-1) and neuropilin-2 were significantly elevated at 1 day post nerve transection; junctional adhesion molecule-A and Nope were significantly elevated at 7 days after PNI; FGF-BP, Neuropilin-1 and Neuropilin-2 were increased at 14 days post nerve transection; and only MCP-1 was increased at 28 days post nerve transection.

Differences in biological processes in the proximal and distal stumps after sciatic nerve injury

The differences in the expression of upregulated cytokines might potentially impact peripheral nerve regeneration. We therefore performed functional analysis to elucidate the roles of the different cytokines in the proximal and distal nerve stumps (**Figure 6A–D**). Interestingly, there were some differences in biological processes between the proximal and distal nerve stumps. For example, the P-value of the inflammatory response in the distal stumps was greater than that in the proximal nerve stumps, as were those for the apoptotic response and cell–cell adhesion. These biological processes might occur more in the distal stumps and might be associated with WD (demyelination, macrophage aggregation and myelin debris elimination). However, the *P*-value for angiogenesis (sprouting angiogenesis and the VEGF receptor signaling pathway) in the proximal stumps was greater than that in the distal nerve stumps at 1, 7 and 14 days post nerve transection.

To clarify the relationships between axons and upregulated cytokines in the proximal and distal nerve stumps, we performed membership analysis with Metascape (**Figure 6E–H**). The search term "axon" was applied to ontologies, including GO Biological Processes and KEGG Pathways. The number of cytokines related to axons in the distal nerve stumps was greater than that in the proximal nerve stumps, while the percentages of cytokines related to axons showed distinct patterns. In the proximal nerve stumps, the cytokine percentages related to axons increased from 54.55% to 72.73%, and then decreased from 72.73% to 62.50%. In contrast, in the distal nerve stumps, the cytokine percentages dropped from 57.14% to 51.85%, and then increased to 63.64%. These results suggest that axonal events are more common in all biological processes in the proximal stumps than in the distal nerve stumps.

Discussion

Complex processes are involved in regeneration after PNI. In the distal nerve stump, WD, a series of intricate cellular and molecular events, starts after injury. Among these are Schwann cell proliferation, macrophage infiltration and the elimination of axonal debris (Nagarajan et al., 2002). In the proximal nerve stump, the axons disintegrate suddenly, and then regenerate with the help of various cells and the process of angiogenesis. Furthermore, numerous genes and proteins undergo dramatic expression changes and play an essential role in nerve regeneration (Belin et al., 2015). These molecules have distinct functions related to various biological processes in the proximal and distal nerve stumps. A recent study investigated the microenvironment in the distal nerve stump after PNI. However, few studies have attempted a comprehensive analysis of protein expression in the proximal or distal nerve stump.

In this study, we demonstrated, using a protein microarray, that the expression levels of several cytokines change in the proximal nerve stumps. The majority of cytokines were upregulated, while a few were downregulated. These cytokine changes may be closely correlated with nerve regeneration. For example, MCP-1 increases to recruit monocytes and macrophages to the sciatic injury site and participates in inflammatory responses (Niemi et al., 2013). The bioinformatics analyses showed that the responses to sciatic injury were different at the various time points. Axon extension, axon guidance, axonogenesis, sympathetic neuron projection guidance and extension were significantly activated at 1 day post nerve transection. However, immune and inflammatory responses were enriched at 7–14 days post nerve transection, indicating that these responses may not occur immediately, but with a delay in the proximal stump. Subsequently, at 28 days post nerve transection, the neuronal apoptotic process, cell proliferation and response to lipopolysaccharide were involved, which may reflect the removal of cell debris, the proliferation of Schwann cells and the regeneration of axons at this time point. All of these processes and events may be related to classical signaling pathways, including the ERK1/2, Jak-STAT and mitogen-activated protein kinase signaling pathways.

In our previous study, we investigated the changes in cytokines in the distal nerve stumps after sciatic nerve injury (Cheng et al., 2020). To examine the mechanisms underlying the stage- and site-dependence of specific patterns of cytokine expression associated with nerve regeneration, we compared the expression of upregulated cytokines in the proximal and distal stumps at various time points. Interestingly, several interleukins were detected in only the distal stump, and increased expression was observed at 1–14 days post nerve transection for IL-4, IL-1R6, IL-10 and IL-22. However, growth factors and growth factor-related proteins were mainly elevated in the proximal stump, except for nerve growth factor beta, which was upregulated in the distal stump at 1 day post nerve transection.

There were a number of similar changes in cytokines in the proximal and distal stumps. Galectin-1 and Galectin-3 were expressed at high levels at all time points after sciatic nerve injury. Galectin-1 was first identified as a member of a family of β-galactoside-binding lectins that are highly expressed in central and peripheral neurons (Hynes et al., 1990). Galectin-1 induces a switch from the M1 (inflammatory and degenerative) to the M2 (anti-inflammatory and regenerative) phenotype, thereby enhancing macrophage-induced myelin phagocytic capacity (Miron et al., 2013). Within the CNS, Galectin-1 binding to the NRP-1/PlexinA4 complex promotes axonal regeneration and the recovery of locomotor activities following spinal cord injury (Quintá et al., 2014). Within the peripheral nervous system, oxidized Galectin-1 then stimulates macrophages to secrete factors that promote axonal growth and Schwann cell migration, which in turn enhance nerve regeneration (Horie et al., 2004; Quintá et al., 2016). Galectin-3, another β-galactoside-binding lectin family member, is involved in the inflammatory response by recruiting and activating lymphocytes, macrophages and microglia. The inhibition of Galectin-3 suppresses spinal nerve ligation-induced inflammatory processes and attenuates neuropathic pain after PNI (Ma et al., 2016). However, several studies have reported that the absence of Galectin-3 during WD results in augmented inflammation within the nerve microenvironment (including upregulation of Toll-like receptor-2, Toll-like receptor-4, IL-1β and tumor necrosis factor-α) and increases the phagocytic capacity of Schwann cells and macrophages, which ultimately contributes to the key steps of nerve degeneration, including myelin breakdown and clearance (Ferraz et al., 2008; Filer et al., 2009; Mietto et al., 2013).

We believe that the site- and stage-specific expression patterns of the cytokines after nerve injury could be important for nerve regeneration. Therefore, we performed further analyses of these molecules at typical pathway and biological process levels using Metascape analysis. We entered the

gene name lists of the target cytokines and set the various conditions to obtain the results. The Metascape analysis revealed that the *P*-value of the inflammatory response in the distal stump was greater than that in the proximal stump, as were the *P*-values for the apoptotic response and cell–cell adhesion. These results indicate that these biological processes might occur more frequently in the distal stump than in the proximal stump because demyelination, macrophage aggregation and myelin debris elimination take place mainly in the distal stump during WD after PNI. However, angiogenesis is a process that proceeds from the proximal stump to the distal stump. The *P*-value of angiogenesis in the proximal stump was greater than that in the distal stump at 1, 7 and 14 days post nerve transection. Given the importance of axons for nerve regeneration, we performed membership analysis with the term "axon". At 1 day post nerve transection, cytokine percentages of "axon" in the distal stump were higher than those in the proximal stump (57.14% *vs*. 54.55%). In the distal stump, the cytokine percentage was reduced from 1 to 7 days post nerve transection, and then increased from 7 to 28 days post nerve transection. In the proximal stump, however, the trend of cytokine percentages was similar to a parabola, increasing first and then decreasing, which may be because myelin debris need to be immediately cleared in the distal nerve stump after nerve injury. This then allows axons in the proximal nerve stump to regenerate within a permissive microenvironment towards their target organs. Further study is needed to verify the current bioinformatics results for key cytokines and signaling pathways.

In summary, we evaluated the expression levels of cytokines at different stages in the proximal and distal nerve stumps after nerve injury using a protein microarray. After examining the specific expression patterns of cytokines in the proximal and distal nerve stumps, we analyzed the biological processes related to nerve regeneration. Our systematic approach and the visualized results should provide a basis for further investigation into potential therapeutic targets for the clinical treatment of peripheral nerve injuries.

Author contributions: *Study design: XQC, WJX, AJS, YW and AYW; experimental implementation: XQC, XD, GHH, SW, PL, HYM; data analysis: XQC and WJX; manuscript writing: XQC; manuscript revision: AJS, YW, AYW. All authors read and approved the final manuscript.*
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Institutional review board statement: *This study was approved by the the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital (approval No. 2016-x9-07) on September 7, 2016.*
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Additional files:
Additional Table 1: *All KEGG pathways and involved cytokines at 1, 7, 14 and 28 days after sciatic nerve injury.*
Additional Table 2: *All biological function categories and involved cytokines at 1, 7, 14 and 28 days after sciatic nerve injury.*
Additional file 1: *Open peer review report 1.*

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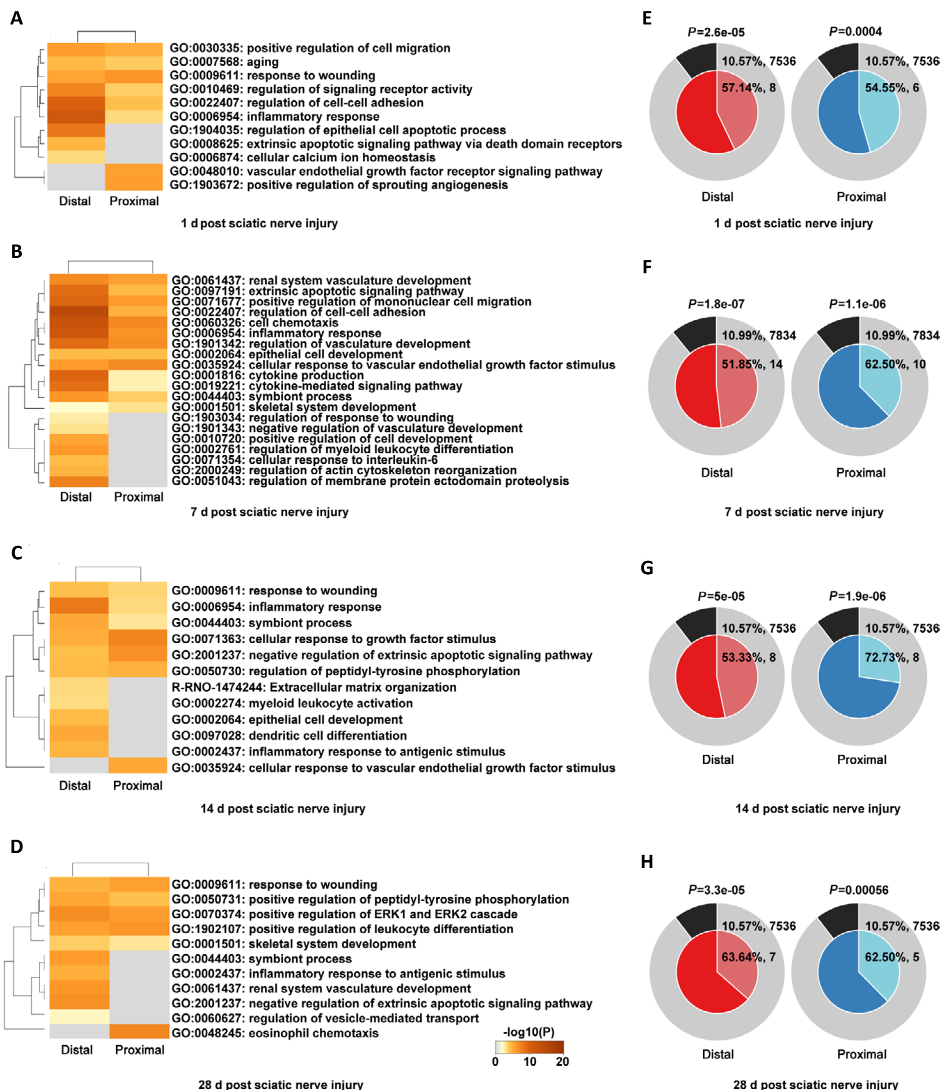

Figure 6 | The difference in enrichment terms between the proximal and distal nerve stumps after sciatic nerve injury analyzed by Metascape.
(A–D) Heatmap showing the top enrichment clusters in both the proximal and distal stumps at 1 (A), 7 (B), 14 (C) and 28 (D) days post nerve transection. Gray indicates no significance. (E–H) Pie chart visualization of the enrichment of membership search terms related to "axon" in the cytokine lists of the proximal stumps and distal stumps at 1 (E), 7 (F), 14 (G) and 28 (H) days post nerve transection. The outer pie shows the number and percentage of proteins in the background related to axon (in black); the inner pie shows the number and percentage of cytokines in our cytokines list related to axon (in red/blue).

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