Characteristics of cytokines in regeneration of injured peripheral nerves

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Abstract
Background
Cytokines are essential cellular modulators of a variety of physiological and pathological activities, including peripheral nerve repair and regeneration. However, the molecular changes of these cellular mediators during peripheral nerve regeneration are not well clarified. The study is aimed to discover critical cytokines for the regenerative process of injured peripheral nerves.

Methods
The sequencing data of the injured nerve stumps and the dorsal root ganglia (DRGs) of Sprague-Dawley (SD) rats subjected to sciatic nerve (SN) crush injury was analyzed to determine expression patterns of genes coding for cytokines. PCR experiments were used to validate the accuracy of sequencing data.

Results
A total of 46, 52, and 54 upstream cytokines were differentially expressed in SNs at 1 day, 4 days, and 7 days after nerve injury. And a total of 25, 28, and 34 upstream cytokines were differentially expressed in DRGs at these time points. The expression patterns of some essential upstream cytokines were displayed in a heatmap and validated by PCR experiment. Bioinformatic analysis of these differentially expressed upstream cytokines after nerve injury demonstrated that inflammatory and immune responses were significantly involved.

Conclusions
In summary, the findings provided an overview of the dynamic changes of cytokines in SNs and DRGs at different time points after rat nerve crush injury, elucidated the biological processes of differentially expressed cytokines, especially the important roles in inflammatory and immune responses during peripheral nerve repair and regeneration, and thus might contribute to identification of potential treatments for peripheral nerve repair and regeneration.

1. Introduction
Peripheral nerves are vulnerable tissues that are generally defenseless to traumatic injuries caused by bump, stretch, crush, and penetrating wounds and non-traumatic injuries caused by genetic,
metabolic, infectious, and medically induced factors (1, 2). Fortunately, unlike nerves in the central nervous system, peripheral nerves can regenerate and achieve certain functional recovery after injury, although fully functional recovery is generally unexpected (3). After peripheral nerve injury, distal nerve stumps undergo Wallerian degeneration, activated Schwann cells and macrophages phagocytosis debris of axon and myelin sheaths, axons of survived neurons regrow toward target tissues for reinnervation (3, 4).

Cytokines are a wide category of immunomodulating proteins or peptides including chemokines, interferons, interleukins, lymphokines, and tumour necrosis factors. Cytokines play essential roles in inflammation and immune responses and participate in the regulation of the maturation, growth, and responsiveness of a variety of cell populations (5, 6). Cytokines have been identified to be constitutively involved in the nervous system in health and disease (7–10). Cytokines are also extremely critical for peripheral nerve injury and repair as fine-tuned expressions of cytokines modulate the cellular behaviors of Schwann cells, macrophages, and neurons and regulate debris clearance, axon growth, and peripheral nerve regeneration (11).

Understanding the molecular changes of these cellular mediators during peripheral nerve regeneration opens new possibilities to improve the repair of injured nerves and to minimize the induction of neuropathic pain (11). On this purpose, previously obtained sequencing data of the injured nerve stumps of Sprague-Dawley (SD) rats subjected to sciatic nerve (SN) crush injury was analyzed to determine expression patterns of genes coding for cytokines (12). Moreover, considering that cytokines retrograde transport to the neuronal bodies and affects neuronal activities, sequencing data of the dorsal root ganglia (DRGs) after rat SN crush injury was also jointly investigated (13). Differentially expressed genes in SNs and DRGs after nerve crush injury were identified and upstream cytokines of these differentially expressed genes were recognized by Ingenuity Pathway Analysis (IPA) bioinformatic tool. Differentially expressed upstream cytokines at 1 day, 4 days, and 7 days after nerve crush injury were subjected to functional enrichment of Gene Ontology (GO) categories and Kyoto Enrichment of Genes and Genomes (KEGG) pathways according to Database for Annotation, Visualization, and Integrated Discovery (DAVID).
2. Materials And Methods

2.1. Sequencing data
RNA deep sequencing data of rat SNs at 0 hour, 1 day, 4 days, 7 days, and 14 days after SN crush injury were conserved in National Center for Biotechnology Information (NCBI) database with the accession number PRJNA394957 (SRP113121). Sequencing data of rat DRGs at 0 hour, 3 hours, 9 hours, 1 day, 4 days, and 7 days after SN crush injury were conserved in NCBI database with the accession number PRJNA547681 (SRP200823). Differentially expressed genes in SNs and DRGs at certain time points after nerve crush injury were selected by comparing their expression levels under the injured status with the expression levels under the uninjured status (0 hour control). Genes with a fold changes < 2 or > -2 and a experimental false discovery rate (FDR) < 0.05 were defined as differentially expressed genes.

2.2. Bioinformatic analysis
Upstream cytokines of differentially expressed genes in SNs and DRGs were identified by IPA bioinformatic tool (Ingenuity Systems Inc., Redwood City, CA, USA) for Ingenuity pathway knowledge base (IPKB)-based upstream regulator analysis. Genes coding for cytokines with a fold changes < 2 or > -2 at 1 day, 4 days, or 7 days as compared with 0 hour were defined as differentially expressed cytokines and were subjected to subsequent bioinformatic analyses.

Commonly differentially expressed cytokines in SNs and DRGs at 1 day, 4 days, or 7 days after SN crush injury were identified by the Venny 2.1.0 online bioinformatic tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html) (14). The expression profiles of these commonly differentially expressed cytokines were demonstrated by a heatmap. Signaling pathways and biological processes involved in differentially expressed upstream cytokines were discovered by DAVID bioinformatic enrichment tools (15, 16).

2.3. Animal surgery and collection of the dorsal root ganglia and SN stumps
The conduction of rat SN crush injury and the collection of SNs and DRGs of uninjured and injured rats were performed as previously described (12, 13). Adult male SD rats weighting 180–220 g were obtained from the Experimental Animal Center of Nantong University (Animal licenses No. SCXK [Su] 2014-0001 and SYXK [Su] 2012-0031) and subjected to animal surgery. Rats were anaesthetized
intraperitoneally with a mixture of 85 mg/kg trichloroacetaldehyde monohydrate, 42 mg/kg magnesium sulfate, and 17 mg/kg sodium pentobarbital. SNs at 10 mm above the bifurcation into the tibial and common fibular nerves were exposed by a skin incision in the left outer mid-thigh. Exposed SNs were crushed with a forceps at a force of 54 N for 3 times with 10 seconds for each time. Rats underwent SN crush injury were sacrificed by decapitation at 1 day, 4 days, and 7 days after animal surgery. Rat underwent sham surgery were sacrificed and designated as 0 hour controls. Rat SNs and lumbar 4 to lumbar 6 DRGs were removed for RNA isolation.

2.4. RNA isolation and PCR validation
RNA was isolated from rat SNs or lumbar 4 to lumbar 6 DRGs using TRizol reagent (Life Technologies, Carlsbad, CA, USA). Isolated RNA samples were reverse transcribed to cDNA using the Prime-Script reagent kit (TaKaRa, Dalian, Liaoning, China) and subjected to PCR experiments using an Applied Biosystems Stepone System (Applied Biosystems, Foster City, CA, USA) with SYBR Premix Ex Taq (TaKaRa) and specific primer pairs of target genes chemokine (C-X-C motif) ligand 10 (Cxcl10) and interleukin 1 receptor antagonist (Il1rn) and reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of primer pairs were as follows: Cxcl10 (forward) 5’-GAAGCACCATAACCAAGGT-3’ and Cxcl10 (reverse) 5’-CAACATGCGACAGGATAGA-3’; Il1rn (forward) 5’-CTTACCTTCAATCGGTCCGA-3’ and Il1rn (reverse) 5’-GATCAGGACGTTGGTGTCAT-3’; and GAPDH (forward) 5’-ACAGCAACAGGTTGTCAG-3’ and GAPDH (reverse) 5’-TTTGAGGGTGCAGCGAACC-3’.

Relative mRNA abundances of Cxcl10 and Il1rn were determined using the comparative $2^{-\Delta\Delta C_{t}}$ method, in which $\Delta C_{t} = C_{t(\text{injured})} - C_{t(\text{uninjured})}$ and $\Delta\Delta C_{t} = C_{t(\text{target gene})} - C_{t(\text{reference gene})}$ (17).

2.5. Statistical analysis
Summarized PCR results were reported as means ± SEM with n = 3. Statistical analysis and graphs were generated using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). The p-value was determined from one-way analysis of variance (ANOVA) and a p-value < 0.05 was considered as statistically significant.

3. Results
3.1. Identification of differentially expressed upstream cytokines in SNs and DRGs following peripheral nerve injury
Previously, the expression patterns of genes in SNs (12) and DRGs (13) at multiple time points after rat SN crush injury were determined and a global view of genetic changes following peripheral nerve injury was obtained. Considering the essential roles of cytokines in tissue remodeling and organ regeneration, IPA bioinformatic analysis was applied to screen upstream cytokines of differentially expressed genes in SNs and DRGs after nerve crush injury. The expression levels of genes coding for these upstream cytokines were further examined and differentially expressed upstream cytokines in SNs and DRGs at 1 day, 4 days, and 7 days after nerve injury were recognized (Table S1).
Table S1

List of differentially expressed upstream cytokines in SNs and DRGs at 1 day, 4 days, and 7 days after rat SN crush injury.

| SN   | DRG   |
|------|-------|
| 1d   | 4d    | 7d   |
| Gene | Log Ratio | Gene | Log Ratio | Gene | Log Ratio | Gene | Log Ratio | Gene | Log Ratio |
| Ccl12 | 14.800 | Ccl12 | 12.409 | Ccl12 | 11.630 | Ccl12 | 7.672 | Il5 | 8.584 | Prh | 8.197 |
| Cxcl2 | 12.446 | Cxcl5 | 9.615 | Cxcl2 | 9.887 | Ilfa2 | 6.993 | Prh | 8.247 | Il24 | 7.583 |
| Cxcl3 | 11.164 | Cxcl2 | 9.041 | Cxcl1 | 9.833 | Iil6 | 6.832 | Cdf40l | 7.196 | Ilfa4 | 7.019 |
| Il10 | 10.552 | Cxcl3 | 8.656 | Cxcl5 | 9.552 | Prl | 5.767 | Iil2 | 7.147 | Il5 | 6.534 |
| Tnfsf14 | 9.445 | Tnfsf14 | 8.654 | Cxcl3 | 9.189 | Iil24 | 4.839 | Ilfa4 | 7.069 | Il6 | 6.316 |
| Il12b | 7.123 | Il10 | 8.317 | Iil17c | 8.328 | Wnt3a | 4.803 | Iil24 | 6.951 | Ilfnb1 | 6.058 |
| Il11n | 6.515 | Il12b | 7.491 | Iil12b | 7.287 | Iil1a | 3.991 | Iil6 | 6.395 | Il9 | 6.037 |
| Csf2 | 6.509 | Cd40l | 7.118 | Iil10 | 7.172 | Ctf2 | 2.669 | Iil2b | 4.247 | Prl | 5.185 |
| Ccl2 | 6.353 | Ilfn | 6.695 | Tnfsf14 | 6.772 | Cxcl14 | 1.256 | Iil1a | 3.430 | Il22 | 5.097 |
| Il1b | 6.263 | Iil1a | 6.565 | Iil2 | 6.317 | Ilfkn | 1.183 | Wnt3a | 2.857 | Csf3 | 4.824 |
| Iil1a | 5.728 | Csf2 | 5.361 | Iilfn | 6.228 | Tnfsf14 | 1.084 | Ccl22 | 1.800 | Wnt3a | 4.391 |
| Ebi3 | 5.203 | Ebi3 | 5.052 | Iil1a | 5.438 | Iil11 | 1.010 | Ccl2 | 1.557 | Iil1a | 3.123 |
| Iil36b | 5.197 | Iil1rn | 4.870 | Wnt3a | 5.218 | Iil12a | -1.086 | Csf1 | 1.481 | Iil12a | 1.775 |
| Ifnb1 | 5.177 | Osm | 4.488 | Iil1rn | 5.205 | Tnfsf10 | -1.385 | Cxcl14 | 1.479 | Iil36rn | 1.672 |
| Ifna4 | 5.139 | Iil36a | 4.383 | Iil17f | 4.911 | Iil17b | -1.501 | Cdf70 | 1.459 | Ccl22 | 1.672 |
| Cxcl1 | 5.127 | Ccl2 | 4.041 | Wnt7a | 4.643 | Ccl19 | -1.501 | Ccl11 | 1.450 | Csf1 | 1.528 |
| Iil1 | 4.997 | Ltf | 3.971 | Ebi3 | 4.258 | Ccl5 | -2.153 | Slurp1 | 1.275 | Cdf70 | 1.409 |
| Iil6 | 4.961 | Crh | 3.921 | Tnfsf14 | 3.650 | Cxcl10 | -2.365 | Lta | 1.137 | Ccl2 | 1.202 |
| Osm | 4.939 | Tnfsf14 | 3.906 | Slurp1 | 3.650 | Tnfsf11 | -2.376 | Iil17c | 1.137 | Iil1b | 1.198 |
| Ccl3 | 4.799 | Iil36b | 3.828 | Ccl3 | 3.494 | Cxcl2 | -3.724 | Tnf | 1.000 | Iil11 | 1.158 |
| Iil2 | 4.762 | Iil6 | 3.658 | Iil1b | 3.375 | Wnt1 | -3.882 | Tnfsf10 | -1.060 | Cxcl5 | 1.087 |
| Tnf | 4.377 | Iilb | 3.496 | Osm | 3.306 | Epo | -4.641 | Crh | -1.267 | Ctf2 | 1.087 |
| Prl | 4.305 | Pfr | 3.458 | Cdf70 | 2.775 | Iil17a | -4.927 | Ccl5 | -1.737 | Ccl11 | 1.065 |
| Iil7f | 3.940 | Iil8 | 3.320 | Cxcl2 | 2.620 | Iil10 | -5.673 | Cxcl10 | -3.079 | Ifkn | 1.035 |
| Wnt3a | 3.926 | Cxcl22 | 3.222 | Ltf | 2.468 | Csf2 | -6.302 | Epo | -4.641 | Iil1rn | 1.010 |
| Ltf | 3.667 | Ccl3 | 3.158 | Pfr | 2.463 | Csf2 | -6.302 | Tnfsf10 | -1.059 |
| Ccl6 | 3.458 | Cxcl10 | 3.070 | Cxcl10 | 2.428 | Iil17b | -7.245 | Iil21 | -1.291 |
| Pfr | 3.436 | Faslq | 2.658 | Iil36b | 2.360 | Cxcl2 | -7.790 | Ccl3 | -1.372 |
| Cxcl10 | 3.109 | Cdf70 | 2.658 | Ccl4 | 2.241 | Tnfsf11 | -2.372 |
| Il18 | 3.093 | Tnf | 2.643 | Tnf | 2.109 | Cxcl10 | -3.337 |
| Timp1 | 3.087 | Iil1 | 2.188 | Cxcl14 | 2.099 | Ccl2 | -3.720 |
| Cdf70 | 2.542 | Ccl6 | 2.173 | Iil8 | 2.037 | Epo | -4.641 | Iil7a | -4.927 |
| Lta | 2.307 | Ccl5 | 2.073 | Iil6 | 1.945 | Iil17a | -4.927 |
| Tnfsf11 | 2.027 | Cxcl1 | 2.052 | Cxcl22 | 1.891 | Csf2 | -6.302 |
| Il17a | 1.805 | Epo | 1.880 | Ccl28 | 1.775 |
| Vav3 | 1.796 | Tnfsf13 | 1.874 | Iil1 | 1.775 |
| Ccl22 | 1.307 | Timp1 | 1.663 | Ccl5 | 1.759 |
| Tnfsf13 | 1.221 | Vav3 | 1.556 | Faslq | 1.422 |
| Iil3 | 1.201 | Tnfsf13b | 1.183 | Ccl6 | 1.412 |
| Aimp1 | 1.111 | Spp1 | 1.089 | Tnfsf13 | 1.282 |
| Namp | 1.063 | Il7 | 1.073 | Vav3 | 1.204 |
| Il21 | -1.121 | Wnt4 | -1.223 | Il17a | 1.191 |
| Cntf | -1.298 | Tnfsf10 | -1.490 | Scgb1a1 | 1.191 |
| Tnfsf10 | -1.749 | Ctf1 | -1.844 | Iil9 | 1.191 |
| Tnfsf15 | -2.474 | Iil6 | -2.032 | Dkk3 | 1.183 |
| Il9 | -4.936 | Tnfsf15 | -2.097 | Cxcl1 | 1.170 |
| Wnt1 | -2.249 | Fam3b | 1.039 | Cntf | -2.480 | Ccl19 | -2.482 | Ccl21 | -3.616 |
Venn diagrams were generated to compare differentially expressed upstream cytokines in SNs and DRGs at certain time points after nerve injury and to obtain a comprehensive view of altered cytokines during nerve regeneration (Fig. 1A-1C). A total of 46 upstream cytokines were differentially expressed in SNs at 1 day after nerve injury. At later time points, a relatively larger number of upstream cytokines were differentially expressed in SNs (Fig. 1D). In DRGs, a smaller group of upstream cytokines were differentially expressed as compared with in SNs. And the numbers of differentially expressed upstream cytokines also increased at later time points after nerve injury (Fig. 1D). Detailed investigation of these differentially expressed upstream cytokines showed that the majority of cytokines were up-regulated and only a few cytokines were down-regulated in SNs. However, in DRGs, the percentage of down-regulated cytokines was much higher (Table S1). The intersection set of Venn diagrams discovered cytokines that were differentially expressed in both SNs and DRGs at the same time points. And the expression changes of these intersected cytokines were listed besides the Venn diagrams (Fig. 1A-1C). Some cytokines, such as interleukin-6 (Il6), were kept up-regulated in SNs and DRGs after nerve injury while some cytokines, such as Cxcl10, were up-regulated in SNs but down-regulated in DRGs (Fig. 1A-1C).

3.2. Demonstration of the expression patterns of upstream cytokines in SNs and DRGs following peripheral nerve injury

To identify the dynamic changes of critical cytokines during nerve regeneration, intersected cytokines in SNs and DRGs were further studied. A total of 27 cytokines were differentially expressed in both SNs and DRGs at 1 day, 4 days, or 7 days after nerve injury. The expression levels of these cytokines were investigated and displayed in a heatmap (Fig. 2). Some cytokines showed similar expression treated in both SNs and DRGs. For example, tumor necrosis factor ligand superfamily member 10 (Tnfsf10) was down-regulated in both SNs and DRGs after nerve injury, CD40 ligand (Cd40lg) was up-regulated in both SNs and DRGs at 4 days after nerve injury, and interleukin-9 (Il9) was up-regulated in both SNs and DRGs at 7 days after nerve injury. Some cytokines, such as Il1rn and C-C motif chemokine ligand 2 (Ccl2), exhibited higher expression changes in SNs as compared with DRGs. The expression patterns of representative cytokines revealed by sequencing assay were further...
validated by PCR experiments. Different batch of SD rats used for sequencing were collected for sciatic nerve crush injury surgery and subsequent PCR validation. Outcomes from PCR experiments demonstrated that the relative abundances of gene coding for cytokine Cxc10 were increased in SNs (Fig. 3A) but decreased in DRGs (Fig. 3B) following nerve injury. And the relative abundances of gene coding for Il1rn were up-regulated in both SNs (Fig. 3C) and DRGs (Fig. 3D). These outcomes were consistent with the expression trends determined by sequencing data (shown in red lines), indicating that sequencing data were of high accuracy.

3.3. Identification of significantly involved signaling pathways of differentially expressed upstream cytokines following peripheral nerve injury

Bioinformatic analyses were performed to evaluate significantly involved signaling pathways of differentially expressed upstream cytokines in SNs and DRGs after nerve injury. Activated signaling pathways that were related to nerve regeneration in up-regulated cytokines and down-regulated cytokines in SNs and DRGs were separately explored (Fig. 4). We focus on upstream cytokines in the current study, so it stands to reason that cytokine-cytokine receptor interaction and chemokine signaling pathway were most strongly enriched signaling pathways. Other significantly enriched signaling pathways included Toll-like receptor signaling pathway, TNF signaling pathway, NOD-like receptor signaling pathway, NF-κB signaling pathway, and JAK-STAT signaling pathway. And these signaling pathways were most robustly involved in up-regulated upstream cytokines in SNs.

3.4. Identification of significantly involved GO biological process categories of differentially expressed upstream cytokines following peripheral nerve injury

Critical nerve regeneration-related biological processes occurred after sciatic nerve crush injury were further discovered by categorizing differentially expressed upstream cytokines to GO terms. Inflammatory response and immune response were the most significantly involved biological processes and were also most strongly involved in up-regulated upstream cytokines in SNs (Fig. 5). Some other inflammatory response and immune response-related biological processes, such as neutrophil chemotaxis, monocyte chemotaxis, cellular response to interleukin-1, also exhibited low p-values, indicating the significance of inflammation and immune response.

To further reveal the importance of inflammation in nerve regeneration, inflammation-related
biological processes were interconnected to a network (Fig. 6A). The inflammation-centered network showed that both acute and chronic inflammatory responses were activated after nerve repair. The chemotaxis, migration, and extravasation of various types of cells, including lymphocytes, macrophages, and monocytes, contribute to activated inflammatory response. Similarly, a network of immune response-related biological processes was also generated (Fig. 6B). Many biological processes related with phenotype modulation of immune cells, such as the activation and proliferation of T cells, B cells, and natural kill cells were significantly participated in the generated network, indicating the critical roles of immune cells in nerve repair and regeneration.

4. Discussion
Peripheral nerve injury induces the disconnection of axons from their cell bodies and leads to the disruption of axons and myelin sheaths in the injured nerve stumps as well as central chromatolysis and nuclear associated changes of somas. With the rapid development of genomics and proteomics, the global genetic and molecular characteristics in a wide variety of physiological and pathological conditions, including peripheral nerve injury and regeneration, were recognized. Many factors were also demonstrated to play fundamental roles in the repair and regeneration of injured peripheral nerves and thus were might be identified as prospective therapies for the treatment of peripheral nerve injury.
Differentially expressed cytokines in the injured SNs might essentially benefit the infiltration and polarization of monocytes, macrophages, and Schwann cells, encourage the phagocytosis and clearance of axon and myelin debris, and promote axon regrowth and regeneration. Actually, a large range of cytokines were found to be up-regulated in the injured nerve stumps. These cytokines might be secreted and released by Schwann cells and macrophages after peripheral nerve injury (18, 19). These up-regulated cytokines, including Ccl2, leukemia inhibitory factor (Lif), tumor necrosis factor-α (Tnf-α), interleukin-1α (Il1-1α), interleukin-1β (Il1-1β), and pancreatitis-associated protein III (Pap-III) recruit the infiltration of monocytes and macrophages into injured nerve sites and contribute to the remodeling and reconstruction of the microenvironment surrounding the injured sites (18, 20–23). In our current study, many other cytokines, including chemokine (C-C motif) ligand 12 (Ccl12), C-X-C
motif chemokine ligand 2 (Cxcl2), and C-X-C motif chemokine ligand 3 (Cxcl3), were found to be particularly high-expressed in the injured nerve stumps after peripheral nerve injury, indicating the potential applications of these cytokines in treating peripheral nerve injury and promoting axon regeneration.

Moreover, it was worth noting that many cytokines might carry out opposing effects at multiple time points during peripheral nerve regeneration and represent a “double-edged sword” (11). Our current study suggested that differentially expressed upstream cytokines in the injured SNs after peripheral nerve injury were highly related with inflammation and immune responses. Therefore, the controversial biological roles of cytokines might be due to the degree and timing of inflammation and immune responses induced by different expression levels of cytokines (11). These results were also consistent with our previous finding that robust immune and inflammatory responses were sustained significantly involved during nerve degeneration and regeneration (24). These outcomes implied that, to achieve orchestrated regulation of cytokines, it was of great importance to obtain an overview of the expression patterns of cytokines in the injured nerve stumps at different time points after peripheral nerve injury.

Besides affecting the injured nerve stumps and reconstructing the regenerative microenvironment, cytokines could influence the expressions of neurotrophins and their receptors and thus could affect the neurite outgrowth of neurons (11). For instance, the addition of interleukin 4 (IL-4) or interferon-γ (IFN-γ) to neurotrophin-4 (NT-4)-treated DRG neurons would increase NT-4-induced neurite outgrowth and the addition of TNF-α to neurotrophin-treated DRG neurons would decrease neurotrophin-induced neurite outgrowth (25). In addition, cytokine induced inflammation and immune response would activate retrograde signaling and might induce the death or survival of DRG neurons (11, 26).

Consequently, in our current study, we also jointly determine the dynamic expression levels of cytokines in DRGs and discovered some significantly changed cytokines, such as interferon alpha 4 (Ifna4), Il6, and interleukin 24 (Il24).

Interestingly, some cytokines, such as Cxcl10, were discovered to be up-regulated in nerve stumps but down-regulated in DRGs after nerve injury. It was shown that Cxcl10 could promote the invasion
of lymphocytes and macrophages and affect myelination in a viral model of multiple sclerosis (27) and could induce neuropathic pain in DRGs after chronic constriction injury (28). Therefore, it was possible that up-regulated Cxcl10 in SNs after nerve injury could contribute to debris clearance in the injured nerve stumps while down-regulated Cxcl10 in DRGs could reduce neuropathic pain. Further functional studies would reveal the specific roles of these cytokines during peripheral nerve repair and regeneration and would provide new targets of the treatment of peripheral nerve injuries.

5. Conclusions
In summary, the findings provided an overview of the dynamic changes of cytokines in SNs and DRGs at different time points after rat nerve crush injury, elucidated the biological processes of differentially expressed cytokines, especially the important roles in inflammatory and immune responses during peripheral nerve repair and regeneration, and thus might contribute to identification of potential treatments for peripheral nerve repair and regeneration.

Declarations

Authors’ contributions
Conceived and designed the experiments: SY HX. Performed the experiments: RZ XG YS. Analyzed the data: RZ XG HX. Contributed reagents/materials/analysis tools: SY HX. Wrote the manuscript: RZ SY HX.

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Availability of data and materials
Availability.

Competing interests
The authors declare that there are no competing interests.
Ethics approval and consent to participate

Animal surgery was ethically approved by the Administration Committee of Experimental Animals, Jiangsu, China and the Institutional Animal Care Guideline of Nantong University and complied with the Guide for the Care and Use of Laboratory Animals approved by the National Institutes of Health.

Consent for publication

Not applicable.

References

1. Caillaud M, Richard L, Vallat JM, Desmouliere A, Billet F. Peripheral nerve regeneration and intraneural revascularization. Neural regeneration research. 2019;14(1):24-33.

2. Campbell WW. Evaluation and management of peripheral nerve injury. Clinical neurophysiology: official journal of the International Federation of Clinical Neurophysiology. 2008;119(9):1951-65.

3. Chen ZL, Yu WM, Strickland S. Peripheral regeneration. Annual review of neuroscience. 2007;30:209-33.

4. Geuna S, Raimondo S, Ronchi G, Di Scipio F, Tos P, Czaja K, et al. Chapter 3: Histology of the peripheral nerve and changes occurring during nerve regeneration. International review of neurobiology. 2009;87:27-46.

5. Zhang JM, An J. Cytokines, inflammation, and pain. Int Anesthesiol Clin. 2007;45(2):27-37.

6. Dinarello CA. Proinflammatory cytokines. Chest. 2000;118(2):503-8.

7. Melik-Parsadanianz S, Rostene W. Chemokines and neuromodulation. Journal of neuroimmunology. 2008;198(1-2):62-8.

8. Lind L, Eriksson K, Grahn A. Chemokines and matrix metalloproteinases in cerebrospinal fluid of patients with central nervous system complications caused by varicella-zoster virus. Journal of neuroinflammation. 2019;16(1):42.
9. Galic MA, Riazi K, Pittman QJ. Cytokines and brain excitability. Front Neuroendocrinol. 2012;33(1):116-25.

10. Zhu H, Wang Z, Yu J, Yang X, He F, Liu Z, et al. Role and mechanisms of cytokines in the secondary brain injury after intracerebral hemorrhage. Progress in neurobiology. 2019;178:101610.

11. Dubovy P, Jancalek R, Kubek T. Role of inflammation and cytokines in peripheral nerve regeneration. International review of neurobiology. 2013;108:173-206.

12. Yi S, Zhang H, Gong L, Wu J, Zha G, Zhou S, et al. Deep Sequencing and Bioinformatic Analysis of Lesioned Sciatic Nerves after Crush Injury. PLoS One. 2015;10(12):e0143491.

13. Gong L, Wu J, Zhou S, Wang Y, Qin J, Yu B, et al. Global analysis of transcriptome in dorsal root ganglia following peripheral nerve injury in rats. Biochemical and biophysical research communications. 2016;478(1):206-12.

14. JC O. VENNY. An interactive tool for comparing lists with Venn Diagrams 2007.

15. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic acids research. 2009;37(1):1-13.

16. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature protocols. 2009;4(1):44-57.

17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.

18. Chen P, Piao X, Bonaldo P. Role of macrophages in Wallerian degeneration and axonal regeneration after peripheral nerve injury. Acta neuropathologica. 2015;130(5):605-18.

19. Liu P, Peng J, Han GH, Ding X, Wei S, Gao G, et al. Role of macrophages in peripheral
nerve injury and repair. Neural regeneration research. 2019;14(8):1335-42.

20. Namikawa K, Okamoto T, Suzuki A, Konishi H, Kiyama H. Pancreatitis-associated protein-III is a novel macrophage chemoattractant implicated in nerve regeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2006;26(28):7460-7.

21. Perrin FE, Lacroix S, Aviles-Trigueros M, David S. Involvement of monocyte chemoattractant protein-1, macrophage inflammatory protein-1alpha and interleukin-1beta in Wallerian degeneration. Brain : a journal of neurology. 2005;128(Pt 4):854-66.

22. Shamash S, Reichert F, Rotshenker S. The cytokine network of Wallerian degeneration: tumor necrosis factor-alpha, interleukin-1alpha, and interleukin-1beta. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2002;22(8):3052-60.

23. Van Steenwinckel J, Auvynet C, Sapienza A, Reaux-Le Goazigo A, Combadiere C, Melik Parsadaniantz S. Stromal cell-derived CCL2 drives neuropathic pain states through myeloid cell infiltration in injured nerve. Brain Behav Immun. 2015;45:198-210.

24. Xing L, Cheng Q, Zha G, Yi S. Transcriptional Profiling at High Temporal Resolution Reveals Robust Immune/Inflammatory Responses during Rat Sciatic Nerve Recovery. Mediators Inflamm. 2017;2017:3827841.

25. Golz G, Uhlmann L, Ludecke D, Markgraf N, Nitsch R, Hendrix S. The cytokine/neurotrophin axis in peripheral axon outgrowth. The European journal of neuroscience. 2006;24(10):2721-30.

26. Dubovy P. Wallerian degeneration and peripheral nerve conditions for both axonal regeneration and neuropathic pain induction. Annals of anatomy = Anatomischer Anzeiger : official organ of the Anatomische Gesellschaft. 2011;193(4):267-75.
27. Liu MT, Keirstead HS, Lane TE. Neutralization of the chemokine CXCL10 reduces inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis. Journal of immunology. 2001;167(7):4091-7.

28. Chen Y, Yin D, Fan B, Zhu X, Chen Q, Li Y, et al. Chemokine CXCL10/CXCR3 signaling contributes to neuropathic pain in spinal cord and dorsal root ganglia after chronic constriction injury in rats. Neuroscience letters. 2019;694:20-8.

Figures

![Diagram](image-url)

Figure 1

Overview of differentially expressed upstream cytokines in SNs and DRGs after SN crush injury. (A-C) Venn diagram of differentially expressed upstream cytokines in SNs and DRGs at (A) 1 day, (B) 4 days, and (C) 7 days after nerve injury. Overlapped cytokines in SNs and DRGs were listed. (D) The numbers of differentially expressed upstream cytokines were listed.
Figure 2
Heatmap of the expression levels of commonly differentially expressed upstream cytokines in SNs and DRGs. The relative expression levels of cytokines in SNs and DRGs at 0 hour, 1 day, 4 days, and 7 days were displayed in colors. Green color indicated down-regulation while red color indicated up-regulation. The expression patterns of representative cytokines revealed by sequencing assay were further validated by PCR experiments. Different batch of SD rats used for sequencing were collected for sciatic nerve crush injury surgery and subsequent PCR validation. Outcomes from PCR experiments demonstrated that the relative abundances of gene coding for cytokine Cxc10 were increased in SNs (Figure 3A) but decreased in DRGs (Figure 3B) following nerve injury. And the relative abundances of gene coding for Il1rn were up-regulated in both SNs (Figure 3C) and DRGs (Figure 3D). These outcomes were consistent with the expression trends determined by sequencing data (shown in red lines), indicating that sequencing data were of high accuracy.
Validation of the expression levels of representative cytokines in SNs and DRGs. (A-B) The relative expression levels of Cxcl10 in (A) SNs and (B) DRGs at 0 hour, 1 day, 4 days, and 7 days after rat SN crush injury. (C-D) The relative expression levels of Il1rn in (C) SNs and (D) DRGs at 0 hour, 1 day, 4 days, and 7 days after rat SN crush injury. The expression levels of Cxcl10 and Il1rn were normalized with GAPDH. Asterisks indicated significant differences (p-value<0.05). Red lines indicated the expression trends revealed by sequencing.
Activated nerve regeneration-related KEGG signaling pathways of differentially expressed upstream cytokines in SNs and DRGs. The sizes of circles indicated the numbers of involved differentially expressed upstream cytokines. Colors indicated the significances of KEGG signaling pathways.
Activated nerve regeneration-related GO biological process categories of differentially expressed upstream cytokines in SNs and DRGs. The sizes of circles indicated the numbers of involved differentially expressed upstream cytokines. Colors indicated the significances of GO biological process categories.
Figure 6

Interaction networks of essential GO biological process categories. (A) Interactions of inflammatory response-related GO biological process categories. (B) Interactions of immune response-related GO biological process categories.

Supplementary Files
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SupplementaryTable S1.xlsx