Characteristics of cytokines in the sciatic nerve stumps and DRGs after rat sciatic nerve crush injury

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Research

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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 after peripheral nerve injury 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 were 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, these 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 after peripheral nerve injury, 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 as well as non-traumatic injuries caused by genetic, metabolic, infectious, and medically induced factors (1, 2). Fortunately, unlike central nerves, 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 clear 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 under various physiological and pathological conditions (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 after peripheral nerve injury opens new possibilities to improve the repair of injured nerves and to minimize the induction of neuropathic pain (11). To deciphers critical molecules that may be beneficial for peripheral nerve regeneration, high-throughput analysis methods, such as RNA sequencing and microarray, have been conducted in our laboratory to determine the gene changes after peripheral nerve injury (12-15). These studies showed that many biological functions, such as cellular behavior, tissue/organ development, and inflammation and immune responses were significantly activated after nerve injury. Considering that cytokines are key molecules that regulate inflammation and immune responses, in the current study, previously obtained sequencing data of the injured nerve stumps of Sprague-Dawley (SD) rats subjected to sciatic nerve (SN) crush injury were analyzed to determine expression patterns of genes coding for cytokines (13). Moreover, considering that cytokines retrograde transport to the neuronal bodies and affect neuronal activities, sequencing data of the dorsal root ganglia (DRGs) after rat SN crush injury were also jointly investigated (16). 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 (13) 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 (16) 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 as previously demonstrated (13, 16).

2.2. Bioinformatic analysis

Differentially expressed genes in SNs and DRGs were uploaded to the IPA bioinformatic tool (Ingenuity Systems Inc., Redwood City, CA, USA) for core analysis. Upstream regulators of these differentially expressed genes were identified using the Ingenuity pathway knowledge base (IPKB)-based upstream regulator analysis. Upstream cytokines were then screened out. 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) (17). 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 (18, 19).

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 (13, 16). A total of 24 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 randomly divided into 4 groups (0 hour, 1 day, 4 days, and 7 days) with 6 rats in each group. Rats were anaesthetizated 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 for 3 times (a period of 10 seconds for each time). At 1 day, 4 days, and 7 days after rat SN crush injury, rats were sacrificed by decapitation. Rats in the 0 hour group were subjected to sham surgery. The 6 rats in each group were divided to 3 replications with 2 rats in each replication for tissue collections. Rat SN segments of 5 mm in length at the crush sites as well as lumbar 4 to lumbar 6 DRGs were harvested 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'-GAAGCACCATGAACCCAAGT-3' and Cxcl10 (reverse) 5'-CAACATGCGGACAGGATAGA-3'; Il1rn (forward) 5'-CTTACCTTCATCCGCTCCGA-3' and Il1rn (reverse) 5'-GATCAGGCAGTTGGTGGTCAT-3'; and GAPDH (forward) 5'-ACAGCAACAGGGTGGTGGAC-3' and GAPDH (reverse) 5'-TTTGAGGGTGCAGCGAACTT-3'. Relative mRNA abundances of Cxcl10 and Il1rn were determined using the comparative $2^{-\Delta\Delta Ct}$ method, in which $\Delta Ct=Ct_{(injured)}-Ct_{(uninjured)}$ and $\Delta\Delta Ct=Ct_{(target\ gene)}-Ct_{(reference\ gene)}$ (20).

2.5. Statistical analysis

Summarized PCR results were reported as means ± SEM with n=3. Graphs were generated using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Kruskall-Wallis was applied for statistical analysis 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 (13) and DRGs (16) 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. Green color indicated down-regulated upstream cytokines while red color indicated up-regulated upstream cytokines.

SN								
1d			4d			7d		
Gene	Log Ratio	FDR	Gene	Log Ratio	FDR	Gene	Log Ratio	FDR
Ccl12	14.800	0.000	Ccl12	12.409	0.000	Ccl12	11.630	0.000
Cxcl2	12.446	0.000	Cxcl5	9.615	0.000	Cxcl2	9.887	0.000
Cxcl3	11.164	0.000	Cxcl2	9.041	0.000	Xcl1	9.833	0.000
Il10	10.552	0.000	Cxcl3	8.656	0.000	Cxcl5	9.552	0.000
Tnfsf14	9.445	0.000	Tnfsf14	8.654	0.000	Cxcl3	9.189	0.000
Il12b	7.123	0.007	Il10	8.317	0.000	Il17c	8.328	0.001
Il1rn	6.515	0.000	Il12b	7.491	0.001	Il12b	7.287	0.003
Csf2	6.509	0.243	Cd40lg	7.118	0.020	Il10	7.172	0.029
Ccl2	6.353	0.000	Ifng	6.695	0.146	Tnfsf14	6.772	0.059
Il1b	6.263	0.000	Il1a	6.565	0.000	Il2	6.317	0.120
Il1a	5.728	0.000	Csf2	5.361	0.531	Ifng	6.228	0.244
Ebi3	5.203	0.000	Ebi3	5.052	0.000	Il1a	5.438	0.000
Il36b	5.197	0.000	Il1rn	4.870	0.000	Wnt3a	5.218	0.029
Ifnb1	5.177	0.481	Osm	4.488	0.000	Il1rn	5.205	0.000
Ifna4	5.139	0.480	Il36a	4.383	0.530	Il17f	4.911	0.241
Cxcl1	5.127	0.000	Ccl2	4.041	0.000	Wnt7a	4.643	0.059
Il11	4.997	0.000	Lif	3.971	0.000	Ebi3	4.258	0.000
Il6	4.961	0.000	Crh	3.921	0.531	Tnfsf11	3.650	0.000
Osm	4.939	0.000	Tnfsf11	3.906	0.000	Slurp1	3.650	0.003
Ccl3	4.799	0.000	Il36b	3.828	0.000	Ccl3	3.494	0.000
Il2	4.762	0.478	Il6	3.658	0.000	Il1b	3.375	0.000
Tnf	4.377	0.000	Il1b	3.496	0.000	Osm	3.306	0.000
Prl	4.305	0.479	Pf4	3.458	0.000	Cd70	2.775	0.061
Il17f	3.940	0.478	Il18	3.320	0.000	Ccl2	2.620	0.000
Wnt3a	3.926	0.242	Ccl22	3.222	0.000	Lif	2.468	0.000
Lif	3.667	0.000	Ccl3	3.158	0.000	Pf4	2.463	0.000
Ccl6	3.458	0.000	Cxcl10	3.070	0.000	Cxcl10	2.428	0.000
Pf4	3.436	0.000	Faslg	2.658	0.000	Il36b	2.360	0.030
Cxcl10	3.109	0.000	Cd70	2.658	0.081	Ccl4	2.241	0.000
Il18	3.093	0.000	Tnf	2.643	0.000	Tnf	2.109	0.000
Timp1	3.087	0.000	Il11	2.188	0.000	Cxcl14	2.099	0.000
Cd70	2.542	0.110	Ccl6	2.173	0.000	Il18	2.037	0.000
Lta	2.307	0.002	Ccl5	2.073	0.000	Il6	1.945	0.000
Tnfsf11	2.027	0.012	Cxcl1	2.052	0.000	Ccl22	1.891	0.000
Il17a	1.805	0.338	Epo	1.880	0.122	Ccl28	1.775	0.152
Vav3	1.796	0.000	Tnfsf13	1.874	0.000	Il11	1.775	0.000
Ccl22	1.307	0.000	Timp1	1.663	0.000	Ccl5	1.759	0.000
Tnfsf13	1.221	0.000	Vav3	1.556	0.000	Faslg	1.422	0.000
Il33	1.201	0.000	Tnfsf13b	1.183	0.000	Ccl6	1.412	0.000
Aimp1	1.111	0.000	Spp1	1.089	0.000	Tnfsf13	1.282	0.000
Nampt	1.063	0.000	Il7	1.073	0.002	Vav3	1.204	0.000
Il21	-1.121	0.014	Wnt4	-1.223	0.022	Il17a	1.191	0.570
Cntf	-1.289	0.000	Tnfsf10	-1.490	0.000	Scgb1a1	1.191	0.213
Tnfsf10	-1.749	0.000	Ctf1	-1.844	0.000	Il9	1.191	0.570
Tnfsf15	-2.474	0.000	Il16	-2.032	0.000	Dkk3	1.183	0.000
Il9	-4.936	0.622	Tnfsf15	-2.097	0.000	Cxcl1	1.170	0.000
			Wnt1	-2.249	0.040	Fam3b	1.039	0.236
			Il21	-2.590	0.000	Timp1	1.013	0.000
			Cntf	-2.722	0.000	Mif	-1.046	0.000
			Il12a	-2.802	0.000	Il16	-1.171	0.000
			Il17a	-3.913	0.574	Tnfsf15	-1.748	0.000

			Csf3	-5.724	0.095	Cntf	-2.480	0.000
						Ccl19	-2.482	0.000
						Ccl21	-3.616	0.000

DRG								
1d			4d			7d		
Gene	Log Ratio	FDR	Gene	Log Ratio	FDR	Gene	Log Ratio	FDR
Ccl1	7.672	0.052	Il5	8.584	0.005	Prlh	8.197	0.090
Ifna2	6.993	0.097	Prlh	8.247	0.074	Il24	7.583	0.000
Il6	6.832	0.000	Cd40lg	7.196	0.019	Ifna4	7.019	0.091
Prl	5.767	0.178	Il22	7.147	0.075	Il5	6.534	0.308
Il24	4.839	0.000	Ifna4	7.069	0.075	Il6	6.316	0.000
Wnt3a	4.803	0.097	Il24	6.951	0.000	Ifnb1	6.058	0.311
Il1a	3.991	0.000	Il6	6.395	0.000	Il9	6.037	0.311
Ctf2	2.669	0.103	Il12b	4.247	0.524	Prl	5.185	0.307
Cxcl14	1.256	0.000	Il1a	3.430	0.000	Il22	5.097	0.559
Ifnk	1.183	0.021	Wnt3a	2.857	0.524	Csf3	4.824	0.311
Tnfsf14	1.084	0.509	Ccl22	1.800	0.000	Wnt3a	4.391	0.169
Il11	1.010	0.121	Ccl2	1.557	0.000	Il1a	3.123	0.000
Il12a	-1.086	0.314	Csf1	1.481	0.000	Il12a	1.775	0.001
Tnfsf10	-1.385	0.000	Cxcl14	1.479	0.000	Il36rn	1.672	0.220
Il17b	-1.501	0.305	Cd70	1.459	0.295	Ccl22	1.672	0.001
Ccl19	-1.501	0.003	Ccl11	1.450	0.000	Csf1	1.528	0.000
Ccl5	-2.153	0.000	Slurp1	1.275	0.029	Cd70	1.409	0.333
Cxcl10	-2.365	0.000	Lta	1.137	0.450	Ccl2	1.202	0.000
Tnfsf11	-2.376	0.036	Il17c	1.137	0.450	Il1b	1.198	0.000
Cxcl2	-3.724	0.002	Tnf	1.000	0.002	Il11	1.158	0.060
Wnt1	-3.882	0.369	Tnfsf10	-1.060	0.000	Cxcl5	1.087	0.493
Epo	-4.641	0.371	Crh	-1.267	0.000	Ctf2	1.087	0.654
Il17a	-4.927	0.371	Ccl5	-1.737	0.001	Ccl11	1.065	0.001
Il10	-5.673	0.368	Cxcl10	-3.079	0.000	Ifnk	1.035	0.049
Csf2	-6.302	0.372	Epo	-4.641	0.356	Il1rn	1.010	0.000
			Csf2	-6.302	0.357	Tnfsf10	-1.059	0.000
			Il17b	-7.245	0.035	Il21	-1.291	0.134
			Cxcl2	-7.790	0.000	Ccl3	-1.372	0.033
						Tnfsf11	-2.372	0.034
						Cxcl10	-3.337	0.000
						Cxcl2	-3.720	0.002
						Epo	-4.641	0.357
						Il17a	-4.927	0.357
						Csf2	-6.302	0.358

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 after peripheral nerve injury, SNs and DRGs intersection cytokines 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 heatmaps (Figure 2). Some cytokines showed similar expression trends 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 quantitative PCR experiments. Independent sciatic nerve crush injury experiments were performed in rats for the collection of SNs and DRGs and the conduction of PCR experiments. Cxcl10, a cytokine whose mRNA expressions were up-regulated in SNs but down-regulated in DRGs and Il1rn according to sequencing data, as well as Il1rn, a cytokine whose mRNA expressions were up-regulated in both SNs and DRGs according to sequencing data, were selected for PCR validation. Outcomes from PCR experiments demonstrated that the mRNA levels of cytokine Cxc10 were increased in SNs (Figure 3A) but decreased in DRGs (Figure 3B) following nerve injury. 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.

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 (Figure 4). Cytokine-cytokine receptor interaction and chemokine signaling were most strongly enriched signaling pathways. Other significantly enriched signaling pathways included Toll-like receptor signaling, TNF signaling, NOD-like receptor signaling, NF-κB signaling, and JAK-STAT signaling. 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 and gene function regulatory networks 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 (Figure 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 responses.

To further reveal the intrinsic link among gene function, we performed a GO analysis on the differentially expressed cytokines in both SNs and DRGs at the same time point, and constructed gene function regulatory networks (GO-Tree) for the significant GO terms (p-value<0.05). The analysis showed that inflammation (Figure 6A) and immune responses (Figure 6B) were induced after peripheral nerve injury. 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, contributed to activated inflammatory response (Figure 6A). The immune-centered network showed that many biological processes related with phenotype modulation of immune cells, such as the activation and proliferation of T cells, B cells, and natural killer cells, were significantly participated in the generated network. It indicated the critical roles of immune cells in nerve repair and regeneration (Figure 6B).

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. Moreover, some molecules that are critical for peripheral nerve repair are discovered by screening differentially expressed genes and/or proteins after nerve injury.

Differentially expressed cytokines in the injured SNs might essentially benefit the infiltration and polarization of monocytes, macrophages, and Schwann cells, encourage the 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 (21, 22). These up-regulated cytokines, including Ccl2, leukemia inhibitory factor (Lif), tumor necrosis factor-α (Tnf-α), interleukin-1α (Il-1α), interleukin-1β (I1-1β), 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 (21, 23-26). 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 expressed at high levels in the injured nerve stumps after peripheral nerve injury, indicating the potential applications of these cytokines in treating peripheral nerve injury and promoting axon regrowth.

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 consistent with our previous findings that robust immune and inflammatory responses were not only activated at the early stage after nerve injury but also remained activated over 14 days after nerve injury (27). 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 (28). In addition, cytokine induced inflammation and immune responses would activate retrograde signaling and might induce the death or survival of DRG neurons (11, 29). Consequently, in our current study, we also jointly determined 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, affect myelination in a viral model of multiple sclerosis (30), and induce neuropathic pain in DRGs after chronic constriction injury (31). Therefore, it was possible that up-regulated Cxcl10 in SNs after nerve injury would contribute to debris clearance in the injured nerve stumps while down-regulated Cxcl10 in DRGs might contribute to the reduction of 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 after peripheral nerve injury, 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 SC ZC YS. Analyzed the data: RZ. Contributed reagents/materials/analysis tools: SY HX. Wrote the manuscript: RZ SY HX.

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Availability of data and materials

Sequencing data of rat SNs and DRGs were conserved in NCBI database with the accession number PRJNA394957 (SRP113121) and PRJNA547681 (SRP200823).

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.

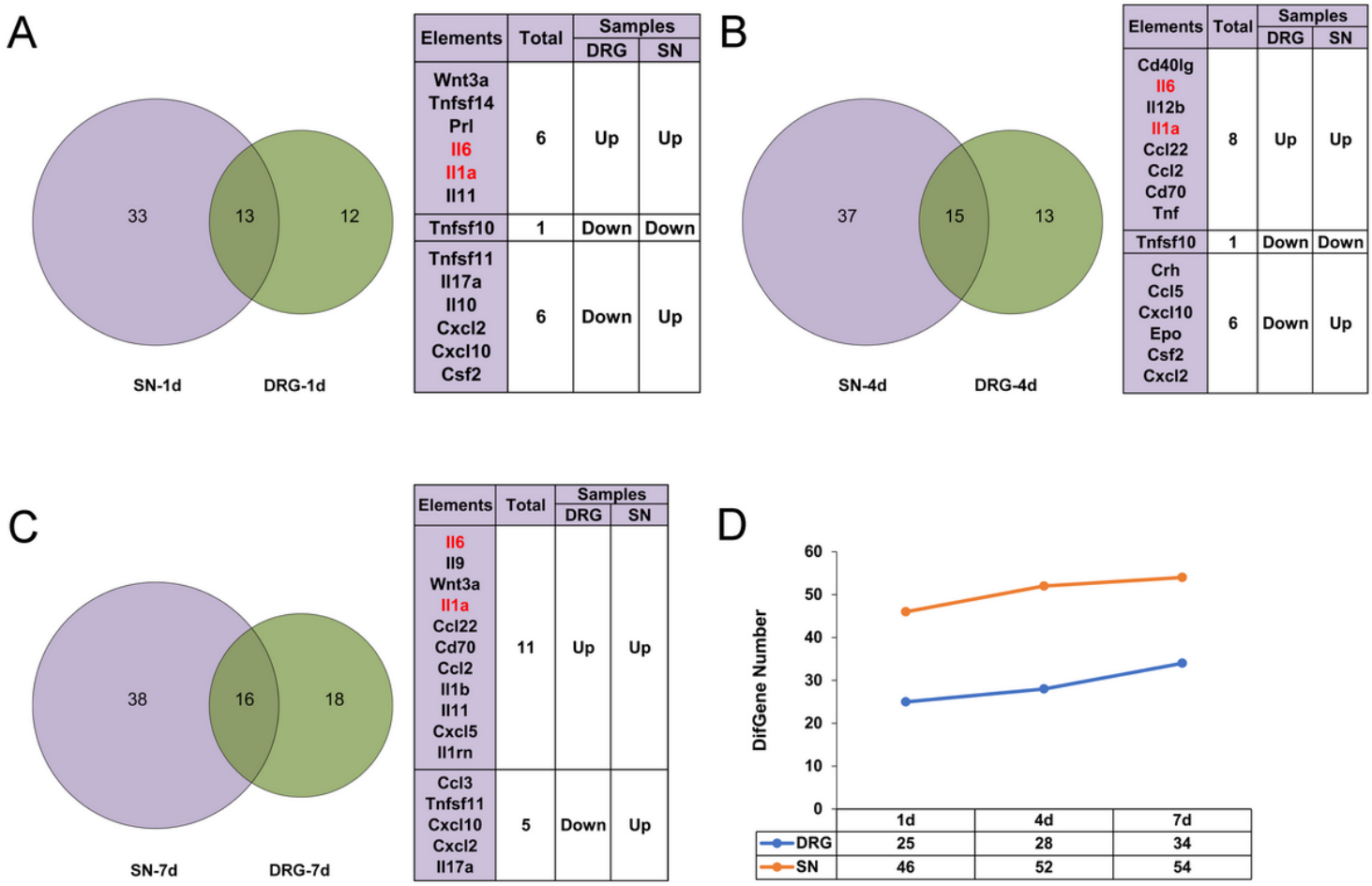
References

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-Parsadaniantz 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. Gong L, Wang D, Zhang L, Xie X, Sun H, Gu J. Genetic changes in rat proximal nerve stumps after sciatic nerve transection. Ann Transl Med. 2019;7(23):763.
13. 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.
14. Yi S, Tang X, Yu J, Liu J, Ding F, Gu X. Microarray and qPCR Analyses of Wallerian Degeneration in Rat Sciatic Nerves. Frontiers in cellular neuroscience. 2017;11:22.
15. Yu J, Gu X, Yi S. Ingenuity Pathway Analysis of Gene Expression Profiles in Distal Nerve Stump following Nerve Injury: Insights into Wallerian Degeneration. Frontiers in cellular neuroscience. 2016;10:274.
16. 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.
17. JC O. VENNY. An interactive tool for comparing lists with Venn Diagrams 2007.
18. 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.
19. 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.
20. 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.
21. 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.
22. 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.
23. 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.

24. 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.
25. 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.
26. 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.
27. 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.
28. 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.
29. 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.
30. 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.
31. 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



Elements	Total	Samples	
		DRG	SN
Wnt3a, Tnfsf14, Prl, Il6, Il1a, Il11	6	Up	Up
Tnfsf10	1	Down	Down
Tnfsf11, Il17a, Il10, Cxcl2, Cxcl10, Csf2	6	Down	Up

Elements	Total	Samples	
		DRG	SN
Cd40lg, Il6, Il12b, Il1a, Ccl22, Ccl2, Cd70, Tnf	8	Up	Up
Tnfsf10	1	Down	Down
Crh, Ccl5, Cxcl10, Epo, Csf2, Cxcl2	6	Down	Up

Elements	Total	Samples	
		DRG	SN
Il6, Il9, Wnt3a, Il1a, Ccl22, Cd70, Ccl2, Il1b, Il11, Cxcl5, Il1rn	11	Up	Up
Ccl3, Tnfsf11, Cxcl10, Cxcl2, Il17a	5	Down	Up

Figure 1

Overview of differentially expressed upstream cytokines in SNs and DRGs after SN crush injury. (A-C) Venn diagrams 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. Red color indicated up-regulated genes at all tested time points. (D) The numbers of differentially expressed upstream cytokines were listed.

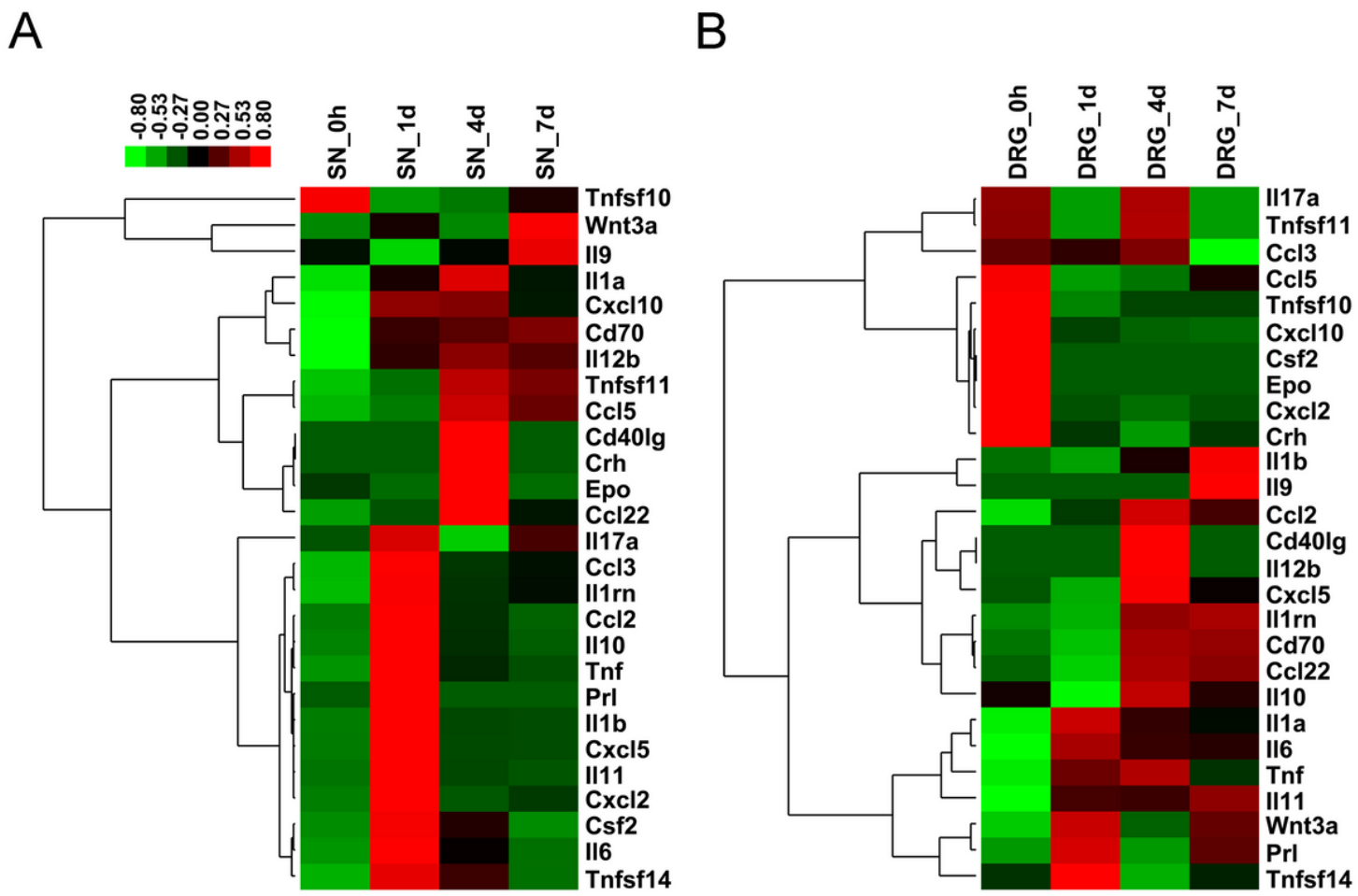


Figure 2

Heatmaps of the expression levels of commonly differentially expressed upstream cytokines in SNs and DRGs. The relative expression levels of cytokines in (A) SNs and (B) 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.

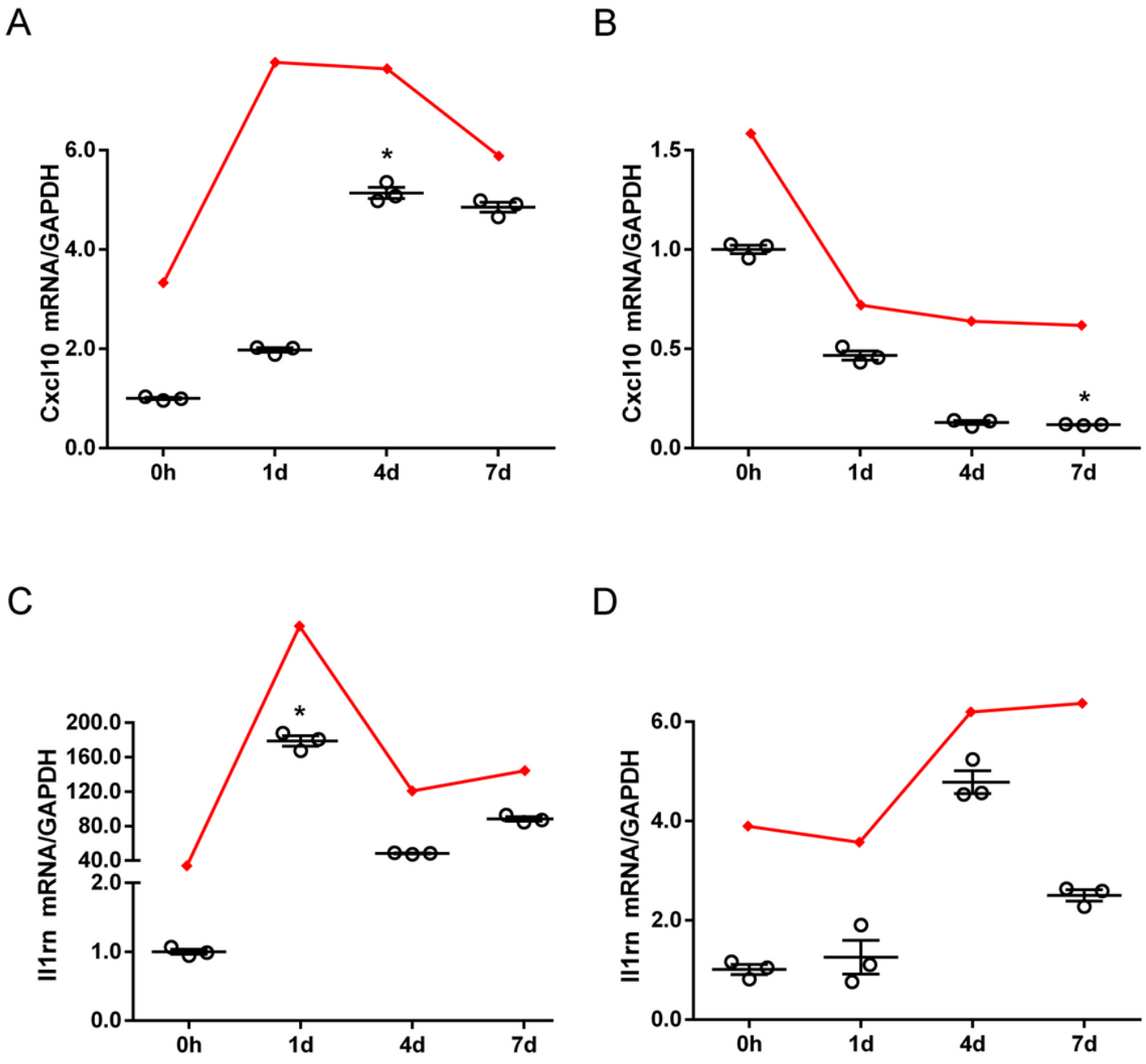


Figure 3

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.

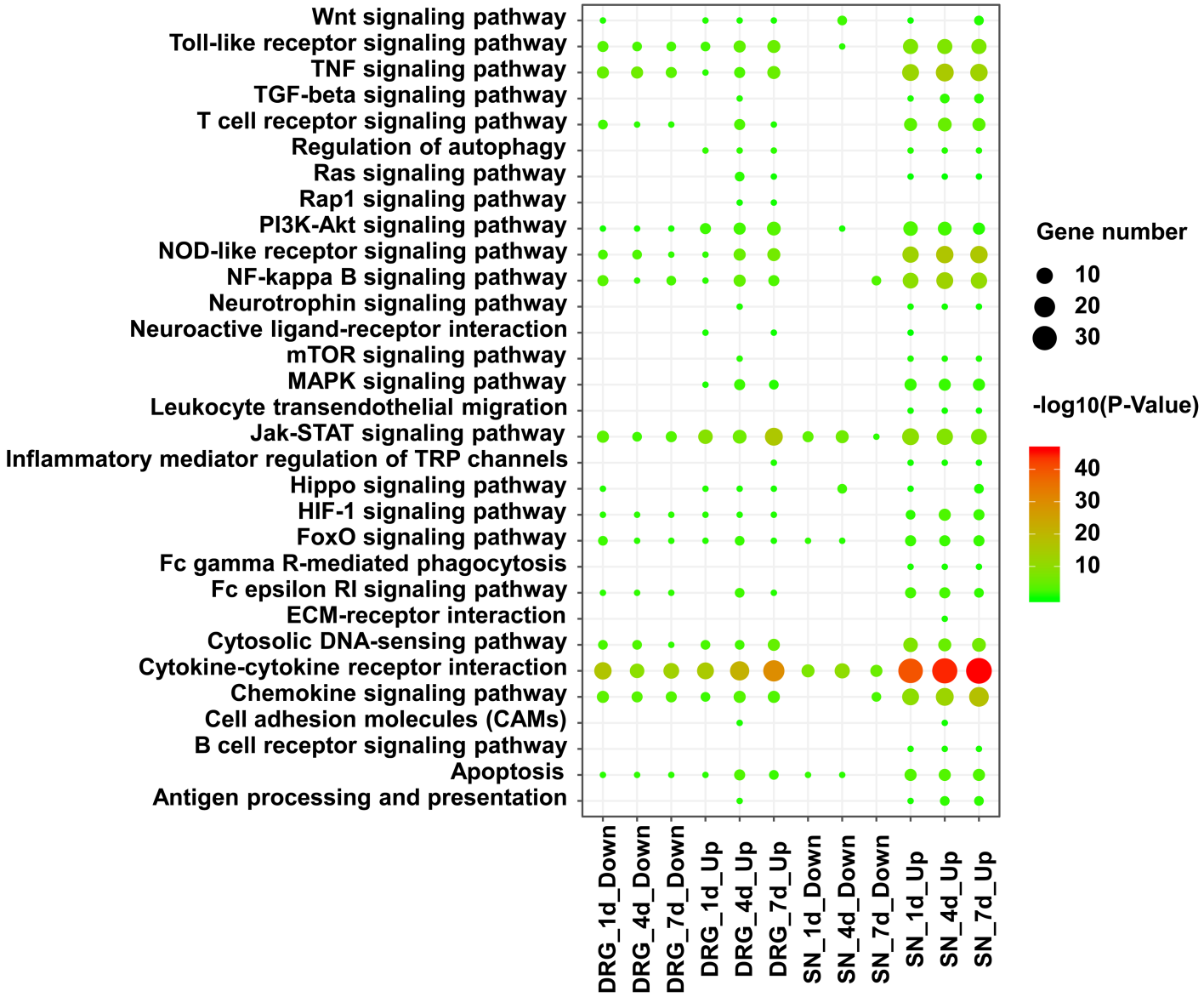


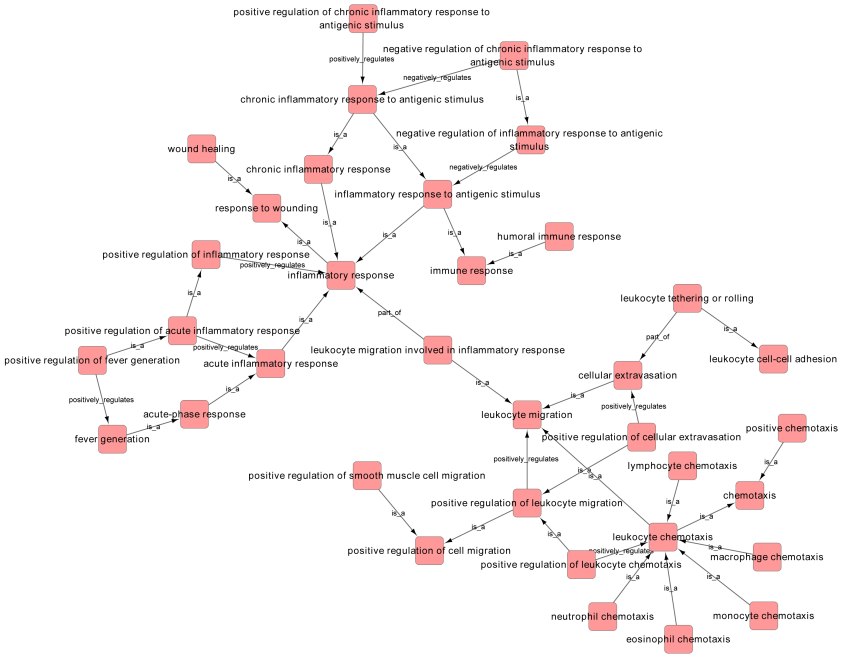
Figure 4

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.

Figure 5

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.

A



B

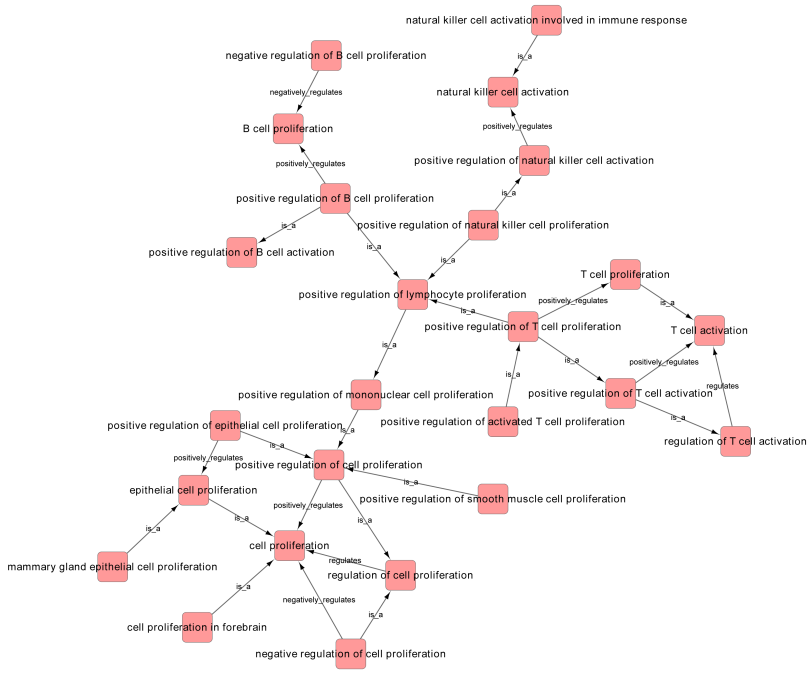


Figure 6

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

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1.xlsx