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Original Article

Profiling of the dynamically alteredgene expression in peripheral nerve injury using NGS RNA sequencing technique

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Abstract: Functional recovery of peripheral nerve injuries is of major demand in clinical practice worldwide. Although, to some extent, peripheral nervous system can spontaneously regenerate, post-injury recovery is often associated with poor functional outcome. The molecular mechanism controlling the peripheral nerve repair process is still majorly unclear. In this study, by utilizing the Next Generation Sequencing (NGS) RNA sequencing technique, we aim to profile the gene expression spectrum of the peripheral nerve repair. In total, we detected 2847 were differentially expressed at day 7 post crush nerve injury. The GO, Panther, IPA and GSEA analysis was performed to decipher the biological processes involving the differentially expressed genes. Collectively, our results highlighted the inflammatory response and related signaling pathway (NFkB and TNFa signaling) play key role in peripheral nerve repair regulation. Furthermore, Network analysis illustrated that the IL10, IL18, IFN-γ and PDCD1 were four key regulators with multiple participations in peripheral nerve repair and potentially exert influence to the repair process. The expression changes of IL10, IL18, IFN-γ, PDCD1 and TNFSF14 (LIGHT) were further validated by western blot analysis. Hopefully, the present study may provide useful platform to further reveal the molecular mechanism of peripheral nerve repair and discover promising treatment target to enhance peripheral nerve regeneration.

Keywords: Peripheral nerve repair, RNA sequencing, inflammation and NFkB

Introduction

Peripheral nerve injuries are common in clinical practice worldwide. However, functional recovery after traumatic injury is often associated with poor results. Unlike substantially limited regenerating capacity of axons in central nervous system (CNS), lesioned axons in the peripheral nervous system (PNS) have the ability to undergo successful regeneration. Recovery following axonal regeneration is largely driven by the ability of neurons to regenerate their axons through the myelin sheath to reinnervate their target organ. Previous studies demonstrated that both neuronal cell bodies and axons undergo a series of pathological events which interact with one another in the whole recovery process [1-4]. Following a nerve injury, downstream axons degenerate in an active process known as Wallerian or anterograde degeneration. The associated Schwann cells dedifferentiate to aprogenitor-like state before proliferation and contribute to the clearance of the axonal and myelin debris. This process is accompanied by robust inflammatory response. The blood-nerve barrier (BNB) is breached and inflammatory cells enter the nerve in large numbers at the damage site [5]. The axons regrow from upstream of damage site guiding themselves back to original target tissues through progenitor-like schwann cell sheath that remain within their basal lamina. Then, progenitor-like schwann cells re-differentiate to fully restore nerve function and the inflammatory response resolves by the completion of the nerve repair [6]. Targeting Schwann cell behavior in nerve repair mechanism, Napoli and colleagues reported the activation of Raf-kinase in myelinated Schwann cells is sufficient to regulate its plasticity in absence of axonal damage. Also, activation of Raf-kinase induces breakdown of the BNB and the influx of inflammatory cells in the peripheral nerve repair site [7]. Furthermore, Grigoryan demonstrated that canonical Wnt signals play a critical role in controlling Schwann cell radial sorting ability in nerve repair process [8]. Although several regulatory signaling pathways have been linked to

aberrations in nerve repair process, systemic knowledge of gene expression profile during peripheral nerve regeneration is still poorly understood.

In the recent decade, Next Generation Sequencing (NGS) technique has been extensively used to study a wide diversity of biological processes, which revolutionized analysis of complex nucleic acid mixtures [9-14]. A single NGS experiment, such as RNA-seq, can reveal the genome-wide identity of millions of sequences in a sample. RNA-Seq is a recently developed approach to transcriptome profiling using deep-sequencing technologies, which provides far more precise measurement of levels of transcripts and their isoforms than other methods [15]. Previous researches have reported certain changes in gene expression and biological processes in the proximal segment of injured sciatic nerves at early time points using DNA microarray analysis [16-22]. In present study, by utilizing NGS RNA-seq technique, we first detected the gene expression spectrum of the peripheral nerve regeneration on crush injury site and demonstrated that inflammatory response and NFkB signaling pathway play a vital role in the peripheral nerve repair mechanism. In addition, we used GO, Panther, IPA and GSEA analysis to identify the biological processes associated with the differentially expressed genes. Expression trends identified in RNA-seq were confirmed using western blot analysis.

Material and methods

Crush nerve injury surgery

Total of six adult male Lewis rats (180-220 g, supplied by Vital River Experimental Animal Corporation) were used in this study. Each 3 rats were used for total RNA and total protein extraction. All experiments were in triplicate. All left side sciatic nerve were crush injured and refer to as injury group (L), while all right intact sciatic nerve tissue serve as control group (R). Each animal was anaesthetized by isoflurane inhalation, and the sciatic nerve was identified and isolated through an incision on the lateral side of the mid-thigh of the left hind limb. A 4 mm width crush injury was made by pinching the nerve with surgical forceps by 90 second at the site just proximal to the division of tibial and common peroneal nerves and the incisions were then closed. All procedures were finished under sterile condition. To alleviate discomfort and pain, painkiller medication were given till day 3. Animals were allowed free access to water and food. All of the animal experimental procedures were performed under the approval of Animal Experiment Ethical Committee of Peking University People's Hospital, where the animals were raised and studied.

Total RNA isolation

The total RNA was isolated using RNeasy Mini Kit (Qiagen 217004) according to manufacturer's protocol. Briefly, all animals were sacrificed on the day 7. Under strict sterile condition, both left side injured nerve and right side control nerve tissue was exposed and isolated from surrounding tissue. The crush injured segment of nerve was easy to distinguish from normal undamaged nerve tissue. Approximately 4mm of injured or normal nerve tissue was resected and used for total RNA isolation.

RNA-Seq by next generation sequencing

Three pairs of total RNA samples were sent to UCLA Clinical Microarray Core Laboratory and all subsequent NGS RNA-seq experiments were finished in UCLA. Libraries for RNA-Seq were prepared with KAPA Stranded RNA-Seq Kit. The workflow consists of mRNA enrichment, cDNA generation, and end repair to generate blunt ends, A-tailing, adaptor ligation and PCR amplification. Different adaptors were used for multiplexing samples in one lane. Sequencing was performed on IlluminaHiSeq2000 for a single read 75 run. Data quality check was done on Illumina SAV. Demultiplexing was performed with Illumina CASAVA 1.8.2 program. The reads were first mapped to the latest UCSC transcript set using Bowtie 2 version 2.1.0 [23] and the gene expression level was estimated using RSEM v1.2.15 [24]. TMM (trimmed mean of M-values) was used to normalize the gene expression. Differentially expressed genes were identified using the edgeR program [25]. Genes showing altered expression with $p<0.05$ and more than 2 fold changes were considered differentially expressed. Significantly different genes were analyzed using the DAVID online analysis tool (http://david.abcc.ncifcrf.gov/) [26]. The Gene Ontology (GO) biological processes were performed and the differentially expressed genes were screened out.

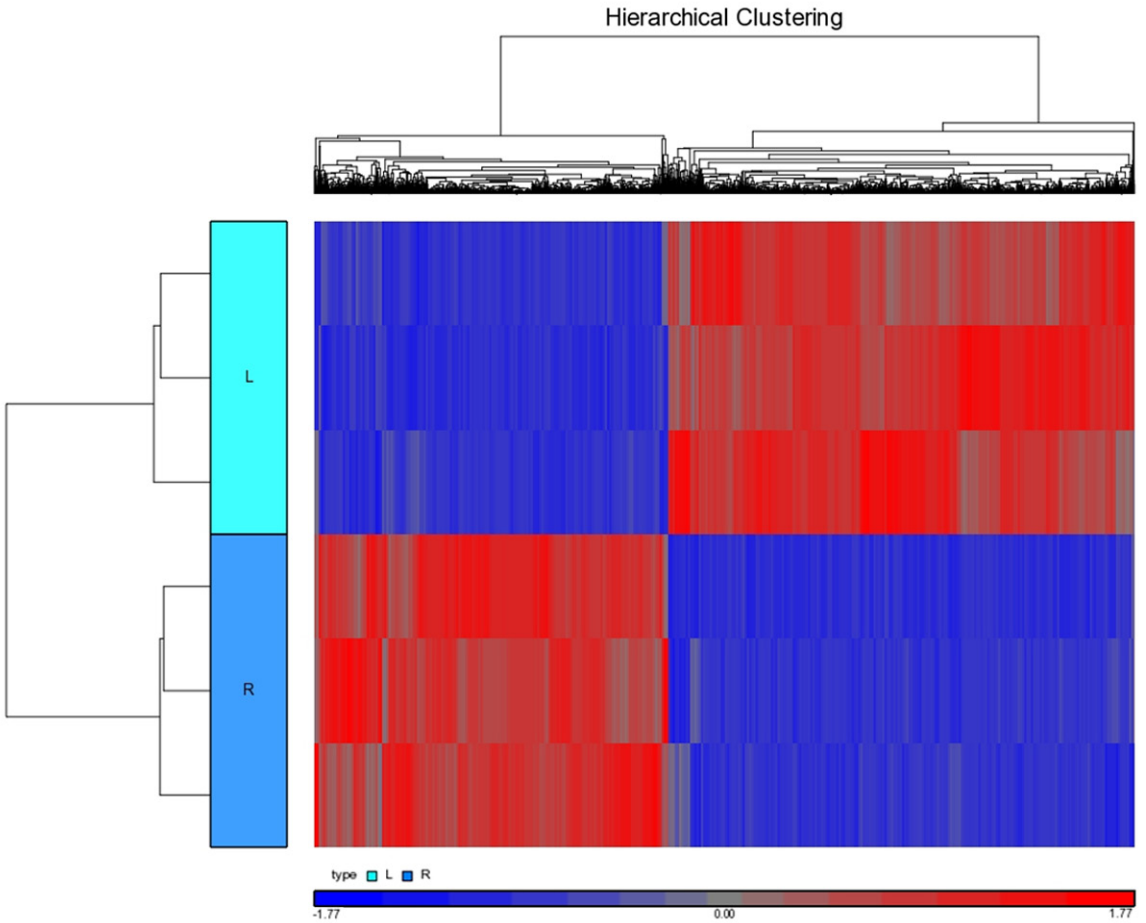


Figure 1. Heat map analysis showing general alteration between nerve injury (L) and control group (R). Our heat map analysis revealed 2847 significant changed genes between L and R group. The two-dimensional hierarchical clustering, using the 2847 genes, clearly showed the degree of separation between L and R group.

Ingenuity pathway analysis (IPA)

The pathway and network analysis was performed using IPA. IPA computes a score for each network according to the fit of the set of supplied focus genes. These scores indicate the likelihood of focus genes to belong to a network versus those obtained by chance. A score > 2 indicates a ≤ 99% confidence that a focus gene network was not generated by chance alone. The canonical pathways generated by IPA are the most significant for the uploaded data set. Fischer's exact test with FDR option was used to calculate the significance of the canonical pathway.

Gene set enrichment analysis (GSEA)

Normalized RNA-seq expression data were pre-ranked based on the fold change between the L samples and R samples. The Hallmark curated Gene sets in MSigDB database 5.0 were used for GSEA analysis [27]. Gene sets were tested for enrichment in rank ordered lists via GSEA using a classic statistics and compared to enrichment results from 1000 random permutations of the gene set to obtain *P* values.

Western blot analysis

Rat peripheral nerve tissues were dissected after perfusion of the animal, liquid nitrogen frozen and lysed in RIPA lysis buffer (500 mMTris, pH 7.4, 150 mM NaCl, 0.5% Na deoxycholate, 1% NP_40, 0.1% SDS and cOmplete protease inhibitors; Roche). Then, tissue lysates were mixed with 4× NuPage LDS loading buffer (Invitrogen), loaded on a 10% SDS polyacrylamide gradient gel (Invitrogen) and subsequently transferred onto a nitrocellulose membrane by a semi-dry transfer apparatus (Bio-Rad). We blocked membranes with 5%

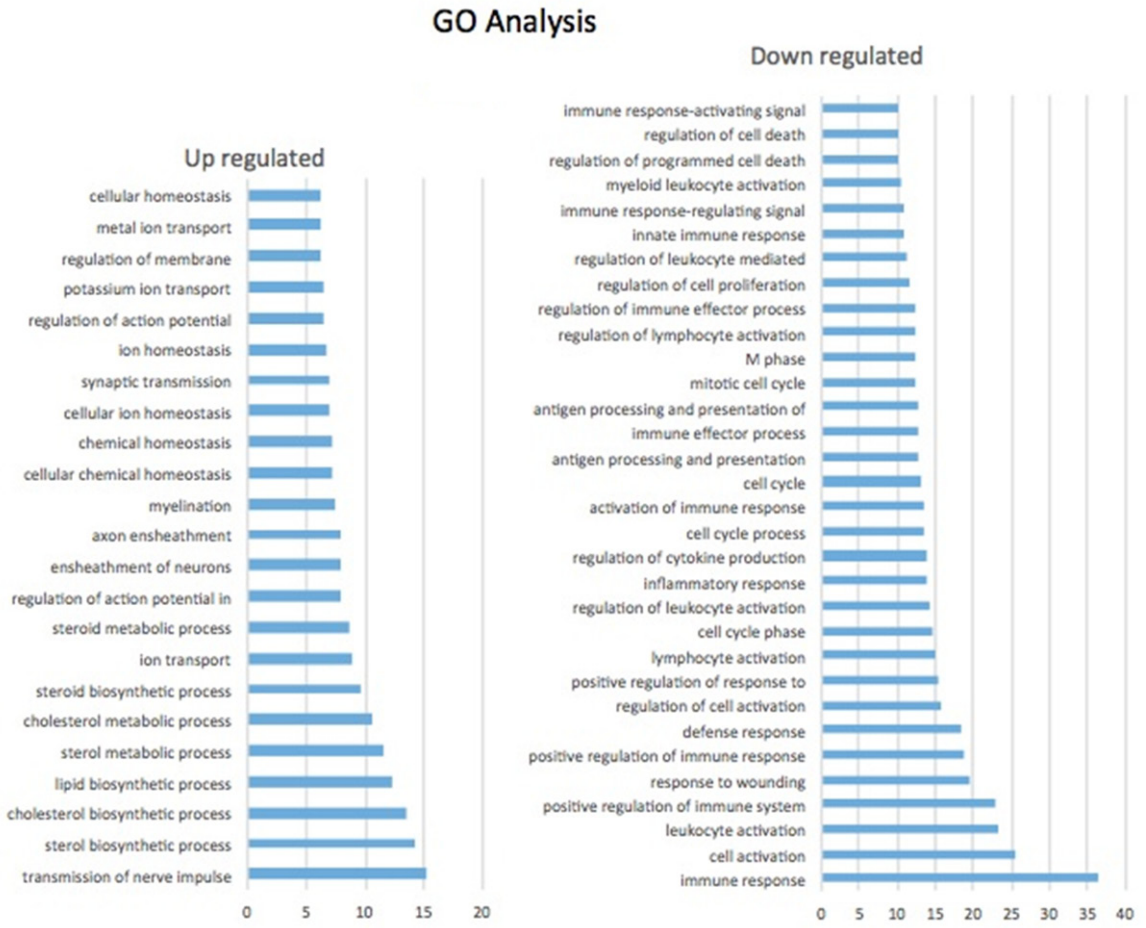


Figure 2. Gene Ontology Enrichment Analysis (GO). We performed GO analysis which address that up regulated genes were associated with 23 classes of biological processes, while down regulated genes were associated with 27 other classes of biological processes.

milk for 1 h and then incubated with primary antibodies overnight. After rinsing, we incubated the immunocomplexes with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega) and visualized the membranes as previously described [28, 29]. We purchased primary antibodies from the following commercial sources: anti-LIGHT (1:1000; sc-28880; Santa Cruz), anti-Pdcd1 (1:1000; sc367266; Santa Cruz), anti-IL10 (1:1000; ab192271; abcam), anti-IL18 (1:1000; ab-191860; abcam) and anti-Interferon gamma (1:1000; ab133566; abcam).

Results

Overview of gene expression profiling in peripheral nerve injury

To uncover molecular mechanisms involved in peripheral nerve regeneration, we first compared the overall genomic profile of crush injured nerve (L group) model and normal nerve (R group) counterpart with normal function using NGS RNA-sequencing (RNA-seq) technique. RNA-seq was performed with the total RNA sample extracted from the crush injury site of sciatic nerves at 7days post nerve crush injury. All the biological replicates (triple repeats) shared similar gene expression pattern based on PCA analysis. Bioinformatics analysis revealed that the expressions of 2847 genes were significantly changed between L and R group (Data not shown). The two-dimensional hierarchical clustering, using the 2847 genes, clearly showed the degree of separation between L and R group (**Figure 1**). Next, we performed Gene Ontology Enrichment Analysis (GO) using the DAVID online tool (**Figure 2**). Up regulated genes were associated with 23 classes of biological processes, such as myelination, axon ensheathment, ensheathment of neurons

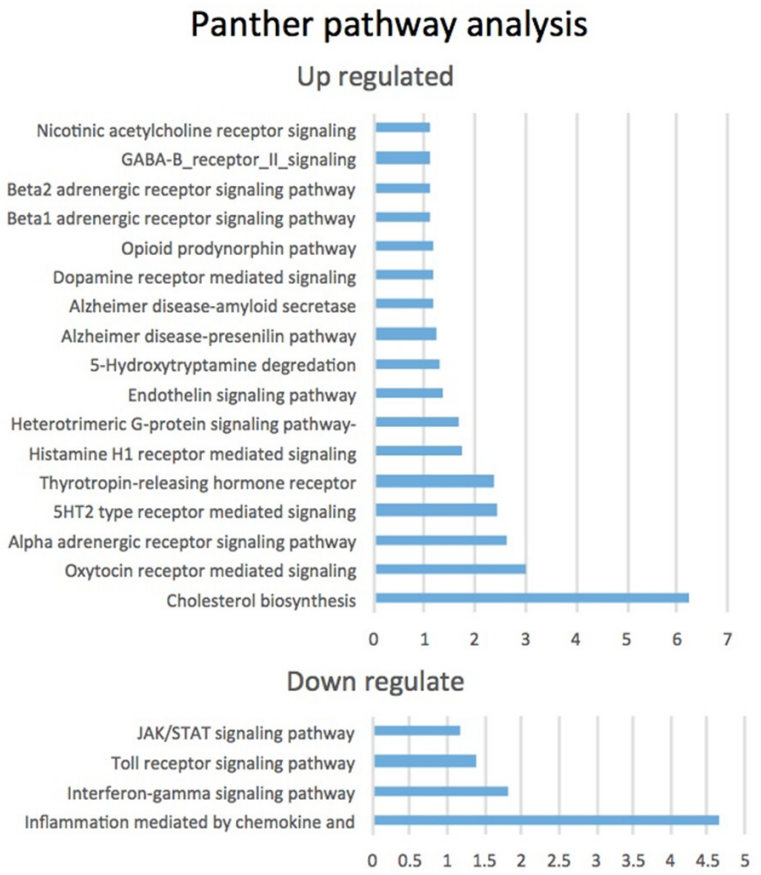
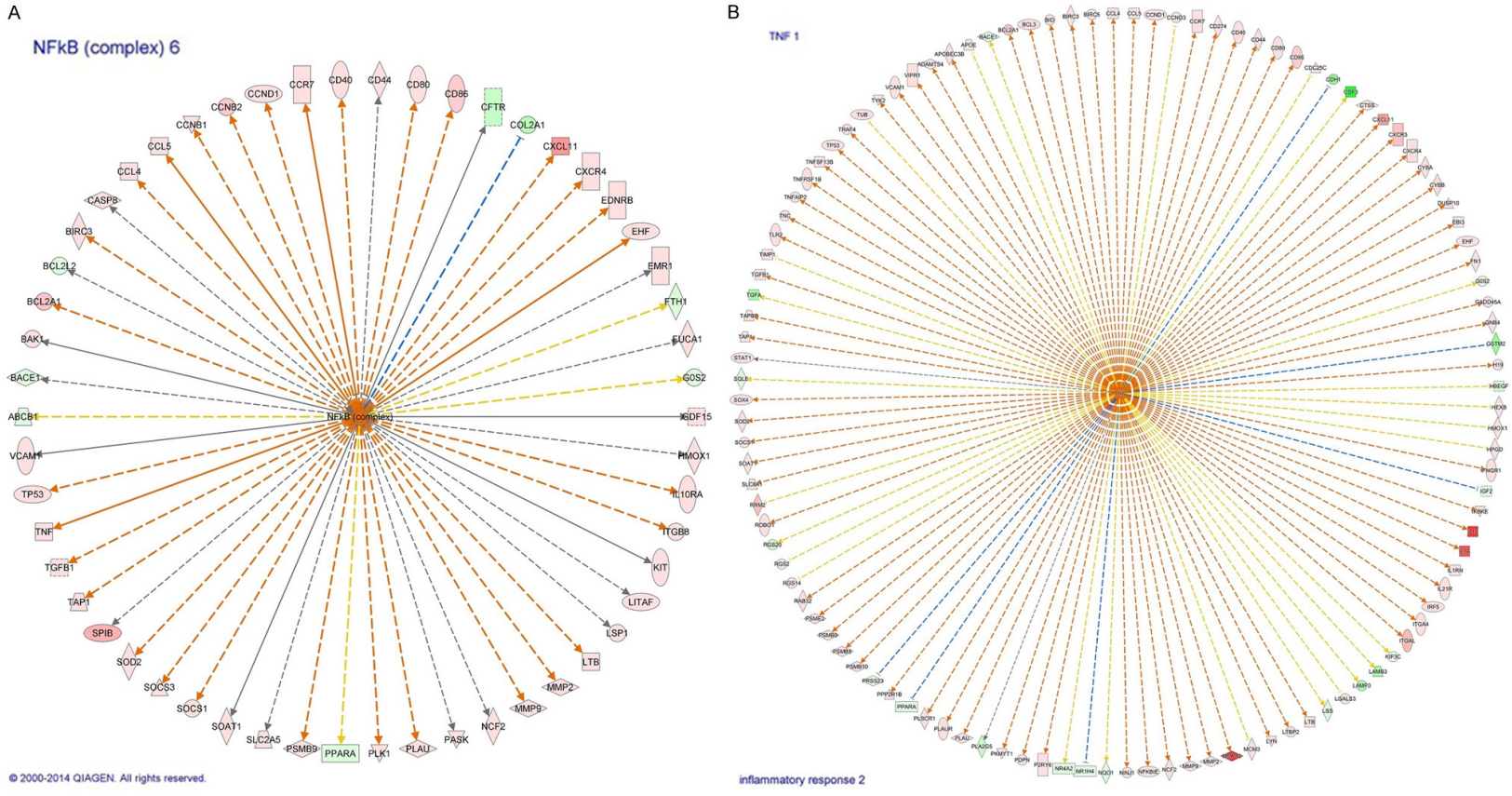


Figure 3. Panther Pathway Analysis. We use Panther Pathway analysis to evaluated peripheral nerve repair regulation on cell signaling level. Up regulated genes were related with 17 pathways, while down regulated genes were only related with 4 pathways.

and nerve impulse transmission etc. While down regulated genes were associated with 27 other classes of biological processes, such as regulation of cell death, immune response-regulating signal, innate immune response, and inflammatory response and wounding response. Moreover, to evaluate the regulation of peripheral nerve regeneration on cell signaling level, we performed Panther Pathway analysis (**Figure 3**). Of note, up regulated genes were related with 17 pathways (higher score means more related), while down regulated genes were only related with 4 pathways. Especially among these pathways, all related genes in 8 signaling pathways namely axon guidance mediated by semaphorins, thyrotropin-releasing hormone receptor pathway, alpha adrenergic receptor signaling, 5HT2 type receptor mediated signaling, oxytocin receptor mediated signaling, heterotrimeric G-protein signaling pathway, cholesterol biosynthesis and inflammation mediated by chemokine were either up or down regulated genes in each of these signaling pathways showing a significant active involvement of these signaling pathways in peripheral nerve repair.

Inflammatory response and NFkB signaling pathway in peripheral nerve repair

Next, we performed Ingenuity pathway analysis (IPA). Data showed that the identified genes were primarily involved in NFkB signaling pathways and inflammatory response (**Figure 4A**). As shown in **Figure 4A**, the majority target genes of NFkB pathway were up regulated which indicated that the NFkB pathway plays an indispensable role in peripheral nerve repair process. In addition, we analyzed TNFa signaling pathway, data demonstrated that the expression of 80 in total 112 TNFa target genes were increased in peripheral nerve repair process, whereas only 6 were inhibited (**Figure 4B**). This finding indicated that the TNFa signaling was largely turned up in the peripheral nerve repair process. Extensive previous studies have shown that TNFa signaling up regulate inflammatory response through NFkB pathway activation which reconfirm our findings. Furthermore, we screened the realm of inflammatory response and also confirmed that most of the upstream regulators were activated and the overall inflammatory response is largely increased (**Figure 4C**). Our result strongly indicate that NFkB mediated inflammatory response play a vital role in the regulation of peripheral nerve repair process, probably also act as a chemo-stimuli agent to guide new axon growth. Next, rigorous Gene set enrichment analysis (GSEA) were also performed to (i) confirm the genome-wide shifts in gene expression in peripheral nerve repair, (ii) reveal how inflammatory response and TNFa pathway targets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GOS2
ISDF15
HMOX1
IL10RA
ITGB8
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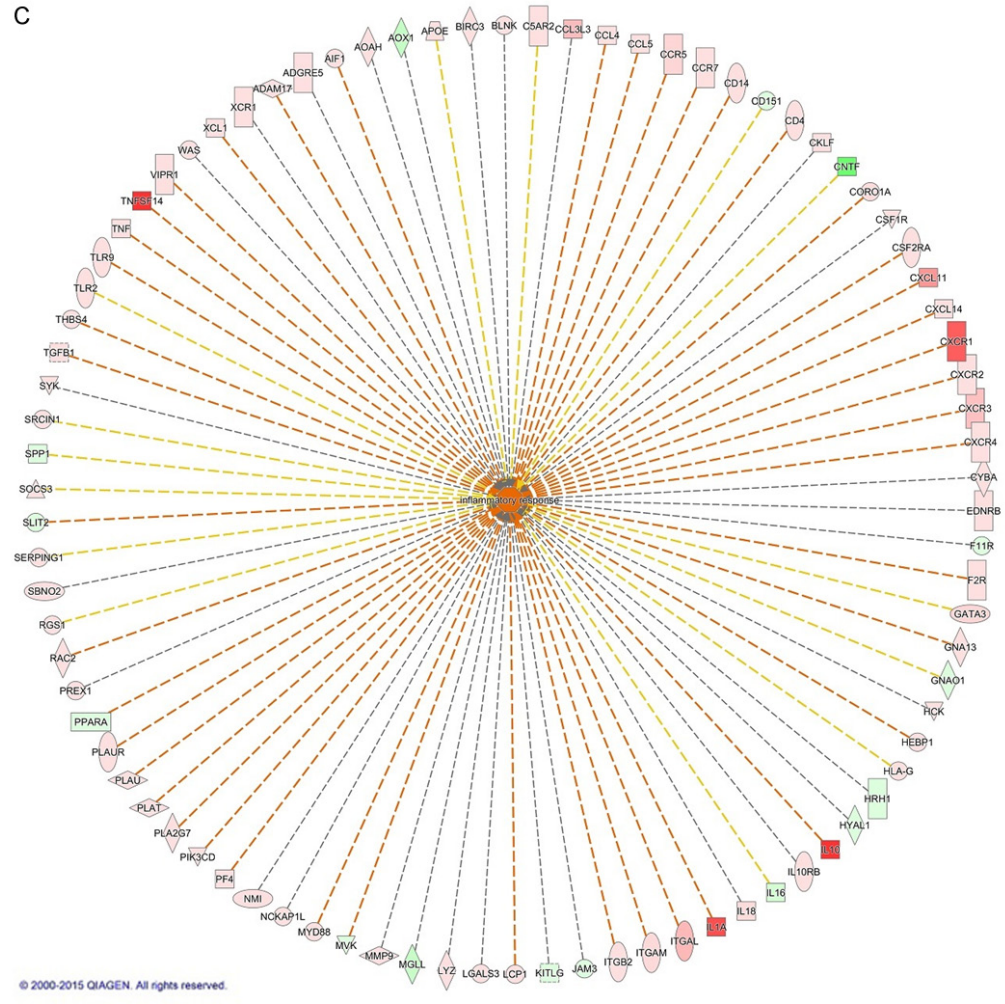

Figure 4. Ingenuity pathway analysis (IPA). Our IPA Data showed that the identified genes were primarily involved in NFkB signaling pathways and inflammatory response. A. Majority target genes of NFkB pathway were up regulated. B. 80 in total of 112 TNFa target genes were increased in peripheral nerve repair process. C. The overall inflammatory response is largely increased.

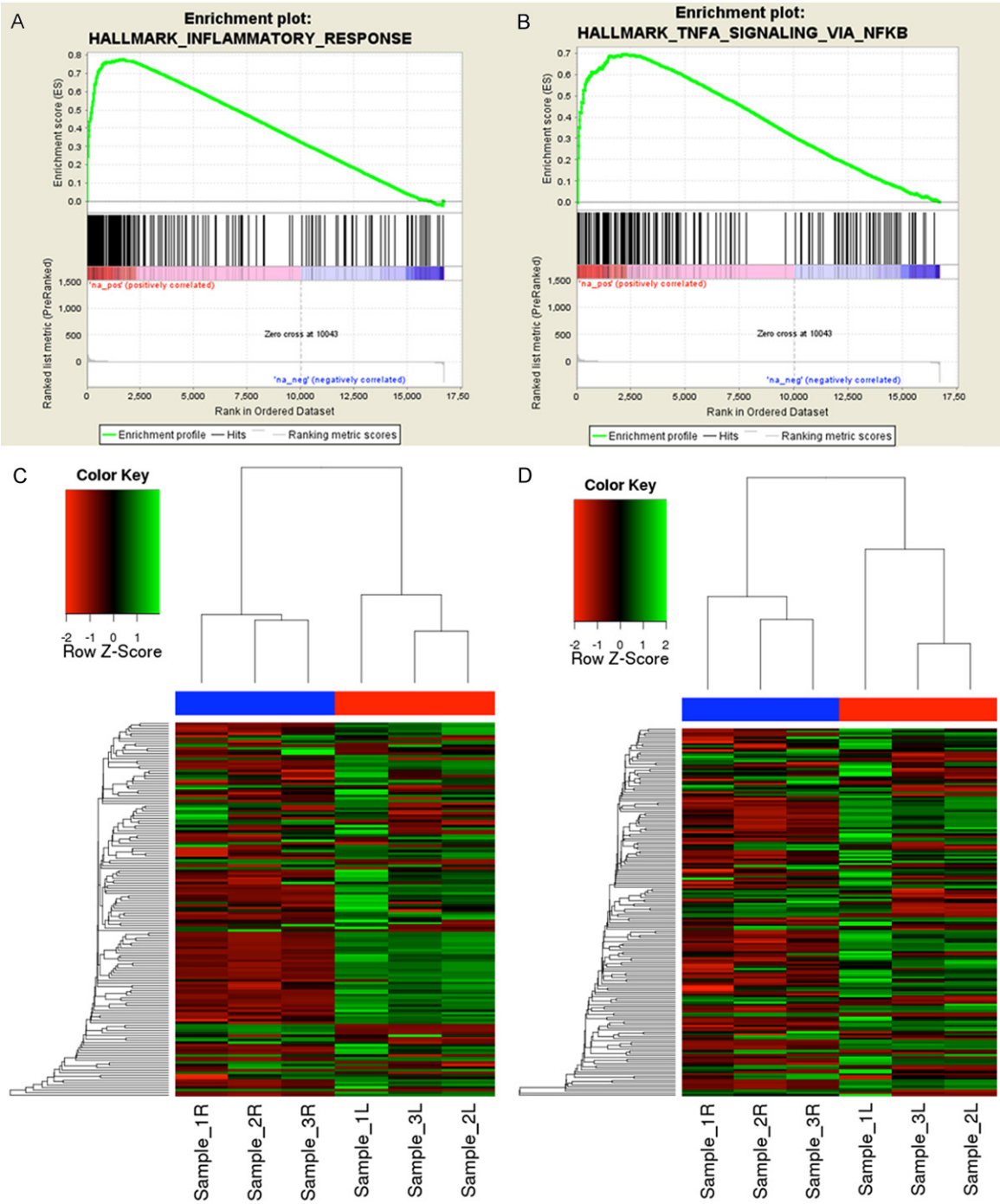

Figure 5. Gene Set Enrichment Analysis (GSEA). GSEA data identified subsets of 191 and 192 genes specific for inflammatory response and TNFa signaling via NFkB, respectively. The Enrichment score was 0.78 and 0.69 with *p*-value at <0.001 and 0.022 respectively.

were regulated at a global level in peripheral nerve regeneration (**Figure 5A**, **5B**). GSEA data identified subsets of 191 and 192 genes specific for inflammatory response and TNFa signaling via NFkB, respectively, both of which were able to specifically distinguish L from R group. Our data demonstrated that peripheral nerve repair is positively correlated with inflammatory response and TNFa signaling via NFkB. The Enrichment score was 0.78 and 0.69 with *p*-value at <0.001 and 0.022 respectively. The FDR q-value was 0.093 and 0.164 respectively.

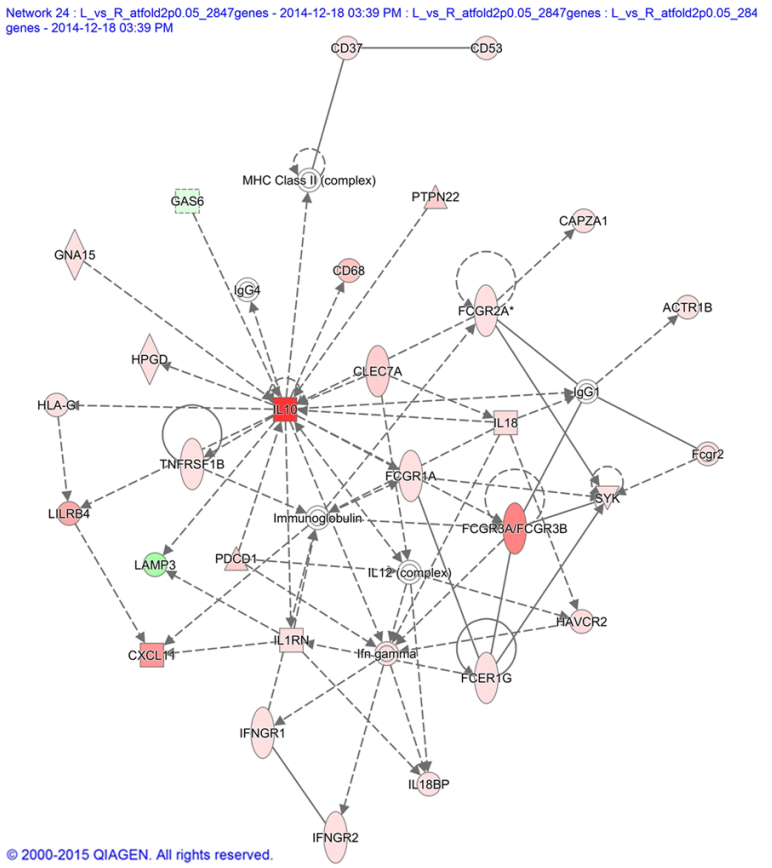

Figure 6. Gene network analysis. We targeted the key "cross-road" regulator in peripheral nerve repair process using gene network analysis. Network analysis illustrated that the IL10, IL18, IFN-γ and PDCD1 were four key regulators with multiple participations in peripheral nerve repair and potentially exert influence to the repair process.

Finally, we utilized gene network analysis with attempt to target the key "cross-road" regulator in peripheral nerve repair process (**Figure 6**). Network analysis illustrated that the IL10, IL18, IFN-γ and PDCD1 were four key regulators with multiple participations in peripheral nerve repair and potentially exert influence to the repair process. Therefore, we targeted these four differentially expressed genes together with TNFSF14 (LIGHT) to verify the RNA-seq data using western blot technique. The results indicated the expression of these five genes exhibited similar expression trends with RNA-seq data (**Figure 7**).

Discussion

In modern world, peripheral nerve injury is common and afflicts individuals from all walks of life. Incomplete recoveries haunt many of these patients, with the ensuing disability resulting in substantial societal and personal costs [30]. Although injured peripheral nerves can regenerate at slow rates of about 1-3 mm per day [31], the regenerative capacity of axons and the growth support of Schwann cells (SCs) decline with time and distance from injury. Therefore, regenerating axons must race against the clock in order to achieve a successful functional recovery [32, 33]. Take clinical brachial plexus injury as an example, the injured neurons must regenerate their lost axons over a distance of as much as a meter to reinnervate the denervated muscles and sensory organs of the hand, a process that can take several years. Within this long time frame, the chronically denervated SCs progressively lose their growth supportive phenotype, becoming atrophic and unable to support regeneration [34-38] and muscles may have atrophied beyond repair. Similarly, prolonged disconnected period of the neurons from the end target also render them less effective in regeneration [32]. As a result, it becomes unlikely that the patient will ever regain hand function or sensation. This problem is even compounded in patients with polytrauma when life-threatening injuries and subsequent complications often delay surgical repair by weeks even months. However, in the recent decades, advances in molecular biology have significantly improved our understanding of the mechanisms of peripheral nerve regeneration. Development of new strategies to enhance peripheral nerve regeneration is urged in clinical practice to have more positive functional recoveries.

After nerve injury, Wallerian degeneration (WD) is the signature pathological process in peripheral nerve repair [39]. Myelin removal is essential for axon regeneration to proceed after

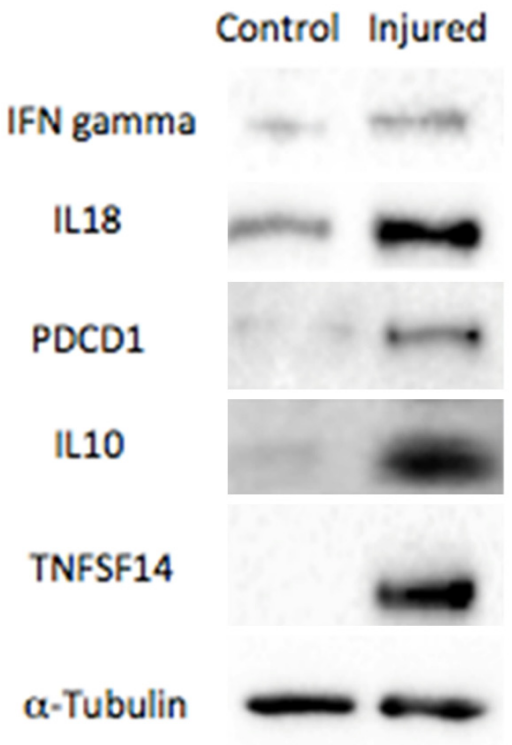

Figure 7. Westernblot verification on crossroad regulator genes. We targeted these four differentially expressed genes together with TNFSF14 (LIGHT) to verify the RNA-seq data using westernblot technique.

nerve injury. A complex interaction exists between macrophages, fibroblasts, SCs, and mast cells expressing inflammatory and anti-inflammatory cytokines during WD [40]. However, mechanisms that terminate the inflammatory response are not well understood. Macrophages switch to an anti-inflammatory phenotype with expression of anti-inflammatory cytokines such as IL-10 [41, 42]. In accordance with our current study, our RNA-seq data showed that IL-10 expression is largely up regulated in the injury site at day 7, and IL-10 play a key role in the regulatory cytokine network in peripheral nerve repair process (**Figure 6**). In addition, our RNA-seq data also targeted a significant up regulation (180 folds change, L vs R) of TNFSF14 (LIGHT) expression in the injured peripheral nerve and act as an important inflammatory response activator in peripheral nerve repair mechanism. Previous work demonstrated that during sciatic nerve injury, LIGHT, expressed by infiltrating B lymphocytes, is required for proper reinnervation and functional motor recovery [43]. Also, activation of the tumor necrosis factor receptor superfamily (TNFRSF) member lymphotoxin beta receptor (LT-bR) by its TNFSF14 ligand triggers a death-signaling pathway in motor neurons [5]. Moreover, LIGHT has been reported to contribute to the selective degeneration of motor neurons in the fatal paralytic disorder amyotrophic lateral sclerosis (ALS) [44, 45]. Interestingly, astrocytes expressing a superoxide dismutase-1 (SOD1) gene mutation are selectively toxic toward motor neurons [46]. This astrocyte-mediated neurotoxicity depends on the production of interferon gamma (IFN-gamma), which leads to an upregulation of LIGHT in motor neurons and commitment of a death program through LT-bR activation. Of interest, our data also detected that IFN-gamma signaling related genes were down regulated from peripheral nerve injury site (**Figure 3**), which may exert neural protective effect via LIGHT regulation in peripheral nerve regeneration. Similarly, IFN-gamma also acts as a vital crossroad regulator in the cytokine network of peripheral nerve recovery (**Figure 6**).

Soon after peripheral nerve injury, a variety of changes occur following WD, including blood-nerve barrier compromise and initiation of cellular changes associated with degeneration. The blood-nerve barrier comprises non-fenestrated endothelial cells connected by tight junctions, and it restricts the movement of proteins, hormones, ions, and toxic substances from blood into neural tissue [47-51]. Although the blood-nerve barrier is already breached at the lesion site after injury, it is firmly intact elsewhere along the nerve until axon degeneration begins. At that point, the barrier is partially compromised along the length of the nerve distal to injury for at least four weeks post-injury [52]. Maximal post-injury perineurial permeability occurs within 4-7 days and corresponds with the peak of the acute inflammatory response [53, 54]. Of interest, these findings coincide with our data in current study that the overall inflammatory response is tuned up in the nerve injury site by post-injury day 7 (**Figure 5A**, **4C**). On the other hand, recent studies have shed light on how Schwann cells and immune cells initially sense injury to nearby axons. Toll-like receptors (TLRs) have been implicated in recognition of tissue damage through binding of endogenous ligands not normally present in the extracellular milieu (e.g., heat shock proteins

[55], mRNA [56], degraded extracellular matrix (ECM) components [57]). Schwann cells express a variety of TLRs. TLR3, TLR4, and TLR7 are constitutively expressed in unstimulated cells and TLR1 is upregulated after axotomy [58]. The expression pattern of TLRs suggests that Schwann cells perform a sentinel role in the PNS. Indeed, peripheral nerve injury induces TLR-dependent changes in activation of transcription factors, cytokine expression, and progression of Wallerian degeneration and functional recovery. For instance, Boivin showed that mice deficient in TLR signaling exhibit reduced pro-inflammatory cytokine expression and macrophage accumulation in distal sciatic nerve, delayed Wallerian degeneration, and impaired functional recovery [59]. Conversely, a single injection of TLR2 or TLR4 ligands expedites myelin clearance and functional recovery. Taken together, these data indicate that endogenous TLR ligands, which are liberated from disintegrating axons, bind TLRs found on Schwann cells and macrophages leading to activation of inflammatory cascades that may be essential for promoting axon regeneration. In our current RNA-seq data, toll receptor signaling pathway related genes were down regulated by post-injury day 7 (**Figure 3**), which indicated that recognition and clearance of the damaged tissue have come to an end and axon regeneration may take over to become the major repair event. All above-mentioned findings explained the reason why we choose investigate the gene profile change in peripheral nerve injury site instead of proximal or distal nerve stamps like some other authors did. Injury site local molecular signaling alteration may act as key chemo-attraction agent to induce axon regeneration and re-myelination through SCs cell fate regulation.

In conclusion, by using NGS RNA-seq technique, we first illustrated the gene expression spectrum change of peripheral nerve on crush injury site. By analyzing the injury site gene profile change, we first demonstrated the inflammatory response and related signaling pathway regulation (NFkB and TNFa signaling) play key role in the nerve repair regulation, which render promising treatment target to enhance peripheral nerve regeneration. Therefore, developing treatments which manipulate inflammatory responses would likely lead to enhanced recovery of patients after peripheral nerve injury.

Acknowledgements

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