Proceedings of Scientific Research Universidad Anáhuac, 2025, 5(9): 5-15.
ISSN-e: 2954-3541.

Original research

The Effect of Immunizing with Neural Derived Peptides on the Expression of Inflammatory Genes Depends on the Severity of Spinal Cord Injury

Elisa García[a,b,1], Raul Silva-García[c], Adrian Flores-Romero[a], Roxana Rodríguez-Barrera[a,2], Yolanda Cruz-Martinez[a,3], Rodrigo Bautista-Román[a], David R. Mayen-Quito[b], Ivan Ignacio Mejía[b], Exsal Manuel Albores-Méndez[b], Marco Antonio Vargas[b], Héctor Faustino Noyola-Villalobos[b], Antonio Ibarra[a,b,4]

[a] Universidad Anáhuac México, Centro de Investigación en Ciencias de la Salud (CICSA), Facultad de Ciencias de la Salud, Estado de México, México.
[b] Secretaría de la Defensa Nacional (SEDENA), Escuela Militar de Graduados en Sanidad, Ciudad de México, México.
[c] Centro Médico Nacional Siglo XXI, Departamento de Inmunología, Ciudad de México, México.

ID ORCID:

[1]https://orcid.org/0000-0001-7588-3846, [2]https://orcid.org/0000-0003-4457-1422, [3]https://orcid.org/0000-0001-5559-2339, [4]https://orcid.org/0000-0003-2489-4689

https://doi.org/10.36105/psrua.2025v5n9.01

ABSTRACT

Introduction: Spinal cord injury (SCI) triggers an inflammatory response that exacerbates tissue damage and limits neuronal regeneration. This response involves the overexpression of inflammatory genes associated with oxidative stress, apoptosis, and demyelination. A promising neuroprotective strategy is immunization with neural-modified peptides (INMP), such as A91 and Cop-1, derived from myelin basic protein. These peptides induce a regulatory immune response via Th2-type T lymphocytes, promoting an anti-inflammatory environment. **Methods:** A severe contusion model of SCI was used in rats. Following injury, animals were immunized with either A91 or Cop-1. Spinal cord tissue was collected seven days post-injury for transcriptomic analysis using microarray technology. Gene expression profiles were compared to a non-immunized control group (PBS). **Results:** Both treatments led to a general reduction in the expression of inflammatory genes. However, only A91 immunization resulted in a statistically significant downregulation of eleven key genes: *Bmp2, Casp1, Casp3, Ccl2, Cebpb, Cish, Socs2, Socs3, Il1rap, Tgfb3,* and *Tnfs11*. These findings are consistent with previous studies from our group, which suggest that the neuroprotective effects of INMPs are less evident in cases of severe SCI. **Conclusion:** Immunization with INMPs modulates the inflammatory response following severe SCI. A91 produced a more pronounced effect than Cop-1, suggesting greater therapeutic potential. These results indicate that gene expression responses to INMPs may vary depending on injury severity, and further research is required to optimize therapeutic strategies.

Key words: gene expression; neural-derived peptides; spinal cord injury.

* *Corresponding Author:* Antonio Ibarra. Universidad Anáhuac México, Centro de Investigación en Ciencias de la Salud (CICSA). Address: Av. Universidad Anáhuac núm. 46, Lomas Anáhuac, 52786. Huixquilucan, Estado de México. Tel.: +52 55 5627 0210. Email: jose.ibarra@anahuac.mx

Received: July 30, 2025.
Accepted: November 21, 2025.

RESUMEN

Introducción: La lesión de la médula espinal (LME) activa una respuesta inflamatoria que agrava el daño tisular y limita la regeneración neuronal. Esta respuesta implica la sobreexpresión de genes inflamatorios, relacionados con procesos como estrés oxidativo, apoptosis y desmielinización. Una estrategia neuroprotectora es la inmunización con péptidos neurales modificados (INMP), como A91 y Cop-1, derivados de la proteína básica de mielina. Estos péptidos inducen una respuesta inmune reguladora mediante linfocitos T tipo Th2, promoviendo un entorno antiinflamatorio. **Metodología:** Se utilizó un modelo de contusión medular severa en ratas. Tras la lesión, los animales fueron inmunizados con A91 o Cop-1. A los siete días post-lesión se obtuvo la médula espinal para análisis transcriptómico mediante microarreglos. Los perfiles de expresión génica se compararon con un grupo control no inmunizado (PBS). **Resultados:** Ambos tratamientos redujeron la expresión general de genes inflamatorios. Sin embargo, solo la inmunización con A91 mostró una disminución estadísticamente significativa en once genes clave: *Bmp2, Casp1, Casp3, Ccl2, Cebpb, Cish, Socs2, Socs3, Il1rap, Tgfb3* y *Tnfs11*. Estos hallazgos coinciden con estudios previos de nuestro grupo, que indican que los efectos neuroprotectores de los INMP son menos evidentes en casos de LME severa. **Conclusión:** La inmunización con INMP modula la respuesta inflamatoria tras LME severa. A91 mostró un efecto más significativo que Cop-1, lo que sugiere un mayor potencial terapéutico. Estos resultados sugieren que la respuesta génica a los INMP varía según la gravedad de la lesión, y se requiere mayor investigación para optimizar las estrategias terapéuticas.

Palabras clave: expresión génica; péptidos de origen neural; lesión medular.

INTRODUCTION

Following spinal cord injury (SCI), several autodestructive mechanisms have been observed. These include significant calcium influx into the cellular compartment, neural fiber damage, metabolic disturbances, destruction of microvessels, and breakdown of the blood-medullary barrier. The latter results in the recruitment of immune cells at the injury site (neutrophils, hematogenous macrophages, and T lymphocytes) and the activation of resident microglia, which triggers an inflammatory reaction at the damaged area, leading to an increased inflammatory response. This inflammatory process is mediated by different cells and proinflammatory cytokines, which exacerbate lipid peroxidation, free radical production, and demyelination, ultimately resulting in extensive secondary tissue damage.[1,2] Furthermore, the exacerbated inflammatory response can trigger a pathological autoreactive reaction, primarily mediated by the activation of T lymphocytes. If these lymphocytes differentiate into a Th1 phenotype (proinflammatory), they contribute to increased demyelination and expansion of the injury. However, if the response is directed toward a Th2 phenotype, the balance Th1/Th2 could regulate secondary damage by developing a neuroprotective microenvironment.[3–5]

The immune response has been demonstrated to play a pivotal role in the pathophysiology of secondary injury.[6] Recently, it has been demonstrated that modulating rather than inhibiting the immune response is beneficial and promotes neurological recovery after SCI.[7,8] Protective autoimmunity (PA) is an innovative strategy based on immune response modulation.[5,9–11] This approach is enhanced by immunization with non-encephalitogenic neural-modified peptides (INMP) such as A91 or Cop-1[3,12] A91 is a myelin basic protein (MBP)-derived peptide (sequence 87-99), obtained by replacing the lysine residue at position 91 with alanine.[12–14] On the other hand, Cop-1 is a random polypeptide synthesized from four amino acids (L-tyrosine, L-glutamic acid, L-alanine, and L-lysine), with an average molar fraction of 0.141, 0.427, 0.095, and 0.0338, respectively.[15,16]

Despite promising results, the precise mechanisms by which PA exerts its neuroprotective effects remain unclear, and many aspects have yet to be elucidated. Gene expression analysis may provide valuable insights into these mechanisms, as gene regulation can influence cellular function over both short and extended periods. Based on our previous studies, the neuroprotective effects of A91 and Cop-1 were found to be limited in cases of severe compared to moderate SCI, we aimed to investigate gene expression changes seven days after severe SCI, following immunization with A91 or Cop-1. Gene expression profiling was performed using microarray analysis to identify novel therapeutic targets associated with INMP in the context of severe spinal cord injury.

https://doi.org/10.36105/psrua.2025v5n9.01

MATERIALS AND METHODS

Study Design

The sample size for this experiment was calculated using an alpha level of 0.05 and a beta of 0.20. Sixteen rats were subjected to severe spinal cord contusion and were randomly assigned to four groups (GraphPad QuickCalcs: http://www.graphpad.com/quickcalcs/): (1) non-immunized rats receiving PBS (n = 4), (2) rats immunized with A91 peptide (n = 4), (3) rats immunized with Cop-1 (n = 4), and (4) Sham-operated rats (n = 4), which were used to normalize gene expression values across all experimental groups. Seven days post-injury, all animals were euthanized, and spinal cord tissue was collected for microarray analysis of inflammation-related gene expression.

Animals

Adult female Fischer 344 rats (F344; 13–14 weeks old, 200–230 g; *n* = 12) were used in three independent experiments. Animals were obtained from Proyecto Camina A.C. and housed according to NIH guidelines for the care and use of laboratory animals. All procedures were approved by the Animal Bioethics and Welfare Committee (Protocol ID: 57204; CSNBTBIBAJ 090812960) and conducted in compliance with the Mexican Official Norm for Laboratory Animal Care (NOM-062-ZOO-1999).

Spinal Cord Injury

Rats were anesthetized with intramuscular ketamine (100 mg/kg; Probiomed, Mexico City) and xylazine (10 mg/kg; Fort Dodge Laboratories, IA, USA). A T9 laminectomy was performed to expose the spinal cord, and a 10 g rod was dropped from a height of 50 mm onto the exposed cord using the New York University (NYU) Impactor to induce a severe SCI.[17,18] After surgery, muscles and skin were sutured in layers. Animals were kept in a temperature-controlled environment at 23°C for the first 7 days post-injury. Manual bladder expression was performed twice daily. Antibiotics (enrofloxacin, 64 mg/kg/day; Marvel, Mexico City) and analgesics were administered daily to prevent infections and minimize pain.

Active Immunization

Sixty minutes after injury, rats were immunized subcutaneously at the base of the tail with a single dose of 150 µg of either A91, Cop-1, or 0.15 M phosphate-buffered saline (PBS), each emulsified in an equal volume of complete Freund's adjuvant (CFA) containing 0.5 mg/mL *Mycobacterium tuberculosis*. A91 (purity >95%) was obtained from Invitrogen Life Technologies (San Diego, CA), and Cop-1 was purchased from Sigma (St. Louis, MO).

Microarray Analysis

Gene expression analysis was focused on 90 inflammation-related genes. Seven days post-injury, a 3 cm spinal cord segment centered at the injury site was collected. Total RNA was extracted using the TRIzol-chloroform method (Sigma-Aldrich), followed by purification using the SuperArrays RT-QPCR grade RNA isolation protocol. RNA concentration and purity were assessed via UV spectrophotometry. Only samples with an A260:A280 ratio of ~2.0 and intact ribosomal bands (28S and 18S) on agarose gel electrophoresis were used.

Complementary RNA (cRNA) was synthesized from 2 µg of total RNA using the TrueLabeling-AMP™ 2.0 Kit (Oligo GEArray®, SuperArrays), incorporating biotin-6-UTP. cRNA was purified using the ArrayGrade™ Cleanup Kit and hybridized to nylon membrane arrays. Each array was performed in quadruplicate. Images were acquired using a cooled CCD camera, and data were analyzed with the SABiosciences GEArray® Expression Analysis Suite. Ribosomal protein L32 and lactate dehydrogenase A (LDHA) were used as internal controls. Expression levels were reported in arbitrary units after normalization. Gene expression from injured tissue was compared to sham-operated controls. A change greater than a 1-fold increase or a decrease of more than 50% was considered significant.

Data Analysis

ImageJ software was used to quantify signal intensity and density (IntDen) for each microarray spot. The same area was selected for all samples using the "rectangle" tool. Expression data from all 90 genes across 15 arrays (three per group) were analyzed. Delta 1 values were calculated

for each gene using LDHA (lactate dehydrogenase A) as a normalization control, and Delta 2 values were derived by comparing results to the Sham group.

Statistical Analysis

Statistical significance was determined using the Kruskal–Wallis test or the Mann–Whitney U test. A p value ≤ 0.05 was considered statistically significant.

RESULTS

To elucidate the molecular mechanisms underlying the neuroprotective effects of A91 and Cop-1, we analyzed the gene expression profiles induced by these synthetic peptides using microarray assays. A broad range of differentially expressed genes was observed in the microarray clustergram across the A91, Cop-1, PBS, and Sham groups (Figure 1).

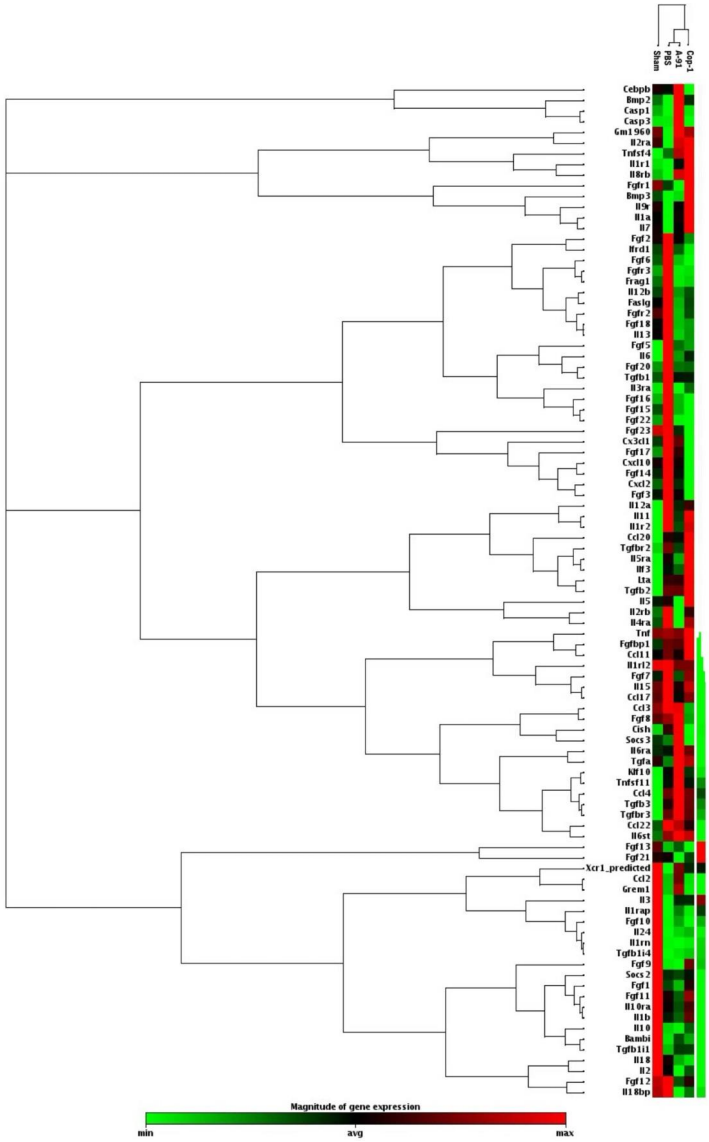

FIGURE 1. Clustergram of inflammatory gene expression following severe SCI in rats immunized with A91 or Cop-1. Colors indicate the relative magnitude of gene expression changes. Several inflammatory genes exhibited differential expression in both the A91 and Cop-1 treatment groups.

https://doi.org/10.36105/psrua.2025v5n9.01

Gene expression changes were systematically analyzed and compared across all experimental groups in the study, with Delta 2 values represented relative to the Sham group. Table 1 summarizes the fold changes in gene expression, as determined by microarray analysis, for each treatment condition. Immunization with A91 or Cop-1 generally resulted in a downregulation of genes associated with inflammatory processes. Notably, statistically significant differences were observed exclusively in the A91-treated group when compared to both the PBS and Cop-1 groups. For clarity, gene expression fold changes in Table 1 are represented using a color-coded scheme: red indicates genes with a ≥1-fold increase, yellow represents genes with a ≥0.3-fold increase, and green denotes genes with a fold change <0.3. Among the A91-treated group, *Bmp2*, *Casp1*, *Casp3*, *Ccl2*, *Cebpb*, and *Cish* exhibited the most pronounced downregulation (average fold change < 0.3), suggesting potential suppression of pro-inflammatory and apoptotic pathways (Table 1).

Table 1. Fold change in gene expression from the microarray analysis across treatment groups

	Groups mean +/- SD			
Gen	PBS	A91	Cop-1	P value
Bmp2	1.76 +/- 0.11	0.28 +/- 0.23	1.04 +/- 0.47	0.001
Bmp3	1.34 +/- 0.38	1.74 +/- 0.45	1.1 +/- 0.28	ns
Casp1	1.38 +/- 0.38	0.26 +/- 0.17	1.04 +/- 0.47	0.001
Casp3	1.45 +/- 0.43	0.29 +/- 0.19	1.15 +/- 0.48	0.05
Ccl2	1.8 +/- 0.04	0.3 +/- 0.17	1.3 +/- 0.14	0.001
Ccl22	1.66 +/- 1.33	1.82 +/- 1.83	1.36 +/- 1.21	ns
Ccl3	1.31 +/- 0.30	0.63 +/- 0.64	0.89 +/- 0.41	ns
Cebpb	1.76 +/- 0.16	0.24 +/- 0.16	1.03 +/- 0.47	0.05
Cish	1.59 +/- 0.11	0.27 +/- 0.14	0.97 +/- 0.38	0.001
Socs2	1.61 +/- 0.29	0.85 +/- 0.24	0.81 +/- 0.13*	0.05
Socs3	1.31 +/- 0.40	0.49 +/- 0.40	0.96 +/- 0.45	0.05
Cx3cl1	1.22 +/- 0.32	0.55 +/- 0.53	0.9 +/- 0.44	ns
Cxcl10	1.32 +/- 0.30	0.51 +/- 0.50	0.94 +/- 0.44	ns
Cxcl2	1.33 +/- 0.29	0.5 +/- 0.48	0.97 +/- 0.43	ns
Il3ra	1.27 +/- 0.26	0.54 +/- 0.45	0.92 +/- 0.41	ns
Fgf1	1.54 +/- 0.49	0.68 +/- 0.48	0.91 +/- 0.34	ns
Fgf10	2.02 +/- 1.50	0.54 +/- 0.11	0.74 +/- 0.12	ns
Fgf11	1.2 +/- 0.41	0.48 +/- 0.30	0.88 +/- 0.39	ns
Fgf12	0.93 +/- 0.26	0.45 +/- 0.11	0.58 +/- 0.10	ns
Fgf13	1.22 +/- 0.40	0.49 +/- 0.45	0.91 +/- 0.51	ns
Fgf14	1.25 +/- 0.29	0.47 +/- 0.42	0.91 +/- 0.44	ns
Fgf15	1.21 +/- 0.30	0.44 +/- 0.37	0.91 +/- 0.42	ns
Fgf16	1.22 +/- 0.24	0.44 +/- 0.38	0.89 +/- 0.41	ns
Fgf17	1.21 +/- 0.34	0.44 +/- 0.35	0.92 +/- 0.46	ns
Fgf18	1.19 +/- 0.36	0.41 +/-0.31	0.87 +/- 0.46	ns
Fgf2	1.11 +/- 0.44	0.38 +/-0.27	0.85 +/ -0.46	ns
Fgf20	0.83 +/- 0.12	0.42 +/-0.20	0.63 +/- 0.04	ns
Fgf21	1.19 +/- 0.39	0.5 +/-0.45	0.9 +/- 0.50	ns

	Groups mean +/- SD			
Gen	PBS	A91	Cop-1	P value
Fgf22	1.18 +/- 0.30	0.46 +/-0.40	0.87 +/- 0.43	ns
Fgf23	1.22 +/- 0.35	0.44 +/-0.38	0.89 +/- 0.47	ns
Fgf3	1.19 +/- 0.37	0.45 +/-0.39	0.89 +/- 0.48	ns
Fgf5	1.11 +/- 0.44	0.45 +/-0.37	0.88 +/ -0.47	ns
Fgf6	1.11 +/- 0.43	0.39 +/-0.30	0.83 +/- 0.48	ns
Fgf7	1.11 +/- 0.45	0.41 +/-0.29	0.83 +/- 0.49	ns
Fgf8	1.19 +/- 0.40	0.45 +/-0.38	0.88 +/- 0.48	ns
Fgf9	1.18 +/- 0.47	0.52 +/-0.39	0.9 +/- 0.47	ns
Fgfbp1	1 +/- 0.23	0.57 +/-0.32	0.74 +/- 0.12	ns
Fgfr1	1.27 +/- 0.17	1.05 +/-0.46	0.94 +/- 0.28	ns
Fgfr2	1.17 +/- 0.33	0.5 +/-0.43	0.85 +/-0.40	ns
Fgfr3	1.17 +/- 0.30	0.48 +/-0.42	0.87 +/-0.40	ns
Frag1	1.16 +/- 0.32	0.45 +/-0.39	0.96 +/-0.41	ns
Ifrd1	1.17 +/- 0.38	0.42 +/-0.35	0.87 +/-0.47	ns
Il18bp	1.26 +/- 0.36	0.49 +/-0.46	0.92 +/-0.48	ns
Il10	1.2 +/- 0.71	0.63 +/-0.37	0.9 +/-0.19	ns
Il10ra	1.21 +/- 0.40	0.51 +/-0.32	0.85 +/-0.35	ns
Il11	1.21 +/- 0.34	0.48 +/-0.39	0.89 +/-0.46	ns
Il12a	1.18 +/- 0.35	0.47 +/-0.39	0.9 +/-0.46	ns
Il12b	1.14 +/- 0.35	0.5 +/-0.44	0.87 +/-0.41	ns
Il13	1.17 +/- 0.37	0.46 +/-0.39	0.88 +/-0.44	ns
Il15	1.2 +/- 0.21	0.61 +/-0.35	0.85 +/-0.27	ns
Il18	1.22 +/- 0.41	0.49 +/-0.44	0.86 +/-0.38	ns
Il1a	1.23 +/- 0.40	0.56 +/-0.49	0.97 +/-0.48	ns
Il1b	1.26 +/- 0.36	0.5 +/-0.41	0.93 +/-0.45	ns
Il1r1	1.23 +/- 0.37	0.51 +/-0.43	0.95 +/-0.47	ns
Il1r2	1.17 +/- 0.33	0.6 +/-0.55	0.92 +/-0.43	ns
Il1rap	1.71 +/- 0.52	0.5 +/-0.08	1.56 +/-0.13	0.05
Il1rl2	1.24 +/- 0.20	0.49 +/-0.22	0.85 +/-0.21	ns
Il1rn	2.03 +/- 2.12	0.65 +/-0.40	0.95 +/-0.58	ns
Il2	1.23 +/- 0.41	0.52 +/-0.42	0.87 +/-0.40	ns
Il24	1.42 +/- 0.62	0.58 +/-0.46	0.98 +/-0.44	ns
Il2ra	1.31 +/- 0.37	0.53 +/-0.44	0.99 +/-0.47	ns
Il2rb	1.26 +/- 0.33	0.52 +/-0.46	0.97 +/-0.43	ns
Il3	1.92 +/- 0.91	1.74 +/-0.91	1.78 +/-0.68	ns
Il4ra	1.17 +/- 0.38	0.46 +/-0.37	0.92 +/-0.46	ns
Il5	1.2 +/- 0.38	0.55 +/-0.47	0.93 +/-0.46	ns
Il5ra	1.2 +/- 0.38	0.5 +/-0.42	0.92 +/-0.47	ns

https://doi.org/10.36105/psrua.2025v5n9.01

	Groups mean +/- SD			
Gen	PBS	A91	Cop-1	P value
Il6	1.19 +/- 0.28	0.52 +/-0.48	0.97 +/-0.41	ns
Il6ra	1.31 +/- 0.34	0.55 +/-0.46	0.98 +/-0.42	ns
Il6st	1.24 +/- 0.33	0.52 +/-0.41	0.94 +/-0.42	ns
Il7	1.27 +/- 0.38	0.51 +/-0.37	0.99 +/-0.45	ns
Il8rb	1.24 +/- 0.39	0.49 +/-0.39	0.96 +/-0.48	ns
Il9r	1.23 +/- 0.41	0.47 +/-0.37	0.95 +/-0.49	ns
Ilf3	1.26 +/- 0.36	0.51 +/-0.46	0.95 +/-0.47	ns
Lta	1.16 +/- 0.39	0.46 +/-0.36	0.88 +/-0.48	ns
Ccl11	1.21 +/- 0.35	0.52 +/-0.43	0.9 +/-0.42	ns
Ccl17	1.2 +/- 0.24	0.57 +/-0.43	0.89 +/-0.29	ns
Ccl20	1.38 +/- 0.85	1.3 +/-0.56	1.41 +/-0.94	ns
Ccl4	1.22 +/- 0.32	0.53 +/-0.43	0.97 +/-0.42	ns
Tgfa	1.29 +/- 0.37	0.47 +/-0.34	0.97 +/-0.45	ns
Tgfb1	0.94 +/- 0.17	0.42 +/-0.15	0.74 +/-0.09	ns
Tgfb1i1	1.34 +/- 0.45	0.51 +/-0.40	1 +/-0.44	ns
Tgfb1i4	1.49 +/- 0.82	0.48 +/-0.34	0.66 +/-0.21	ns
Tgfb2	1.21 +/- 0.38	0.43 +/-0.33	0.9 +/-0.48	ns
Tgfb3	1.32 +/- 0.37	0.32 +/-0.26	1.32 +/-0.05	0.05
Tgfbr2	1.2 +/- 0.28	0.62 +/-0.45	0.96 +/-0.35	ns
Tgfbr3	1.28 +/- 0.35	0.52 +/-0.40	1.02 +/-0.43	ns
Klf10	1.31 +/- 0.39	0.49 +/-0.39	1.03 +/-0.45	ns
Tnf	1.21 +/- 0.30	0.57 +/-0.27	1.02 +/-0.37	ns
Tnfsf11	1.33 +/- 0.41	0.49 +/-0.41	1.01 +/-0.46	0.05
Tnfsf4	1.26 +/- 0.37	0.49 +/-0.37	0.97 +/-0.46	ns
Faslg	1.02 +/- 0.24	0.48 +/-0.21	0.85 +/-0.26	ns

The values represent relative expression levels compared to the Sham group. Statistical analysis was performed using the Kruskal–Wallis test, followed by post hoc Mann–Whitney U tests ($p < 0.05$). SD: standard deviation.

Among all the genes analyzed, only eleven exhibited statistically significant downregulation in the A91-treated group compared to the PBS and Cop-1 groups. Although the Cop-1 group showed a reduction in the expression of these genes, the differences were not statistically significant when compared to the PBS group (Table 2).

Table 2. Changes in gene expression of the different groups

	Groups mean +/- SD			
Gen	**PBS**	**A91**	**Cop-1**	**p compared with all the groups**
Bmp2	1.76 +/- 0.11	0.28 +/- 0.23	1.04 +/- 0.47	0.001
Casp1	1.38 +/- 0.38	0.26 +/- 0.17	1.04 +/- 0.47	0.001
Casp3	1.45 +/- 0.43	0.29 +/- 0.19	1.15 +/- 0.48	0.05
Ccl2	1.8 +/- 0.04	0.3 +/- 0.17	1.3 +/- 0.14	0.001
Cebpb	1.76 +/- 0.16	0.24 +/- 0.16	1.03 +/- 0.47	0.05
Cish	1.59 +/- 0.11	0.27 +/- 0.14	0.97 +/- 0.38	0.001
Socs2	1.61 +/- 0.29	0.85 +/- 0.24	0.81 +/- 0.13	0.05
Socs3	1.31 +/- 0.40	0.49 +/- 0.40	0.96 +/- 0.45	0.05
Il1rap	1.71 +/- 0.52	0.5 +/-0.08	1.56 +/-0.13	0.05
Tgfb3	1.32 +/- 0.37	0.32 +/-0.26	1.32 +/-0.05	0.05
Tnfsf11	1.33 +/- 0.41	0.49 +/-0.41	1.01 +/-0.46	0.05

The values represent relative expression levels compared to the Sham group. Statistical analysis was performed using the Kruskal–Wallis test, followed by post hoc Mann–Whitney U tests ($p < 0.05$). SD: standard deviation.

DISCUSSION

The present study provides insight into the complex and severity-dependent gene expression changes induced by immunization with neural-derived peptides (A91 and Cop-1) following severe spinal cord injury (SCI). Previous work by our laboratory demonstrated the neuroprotective and anti-inflammatory effects of these peptides in moderate SCI models;[7,8,19] however, these effects were significantly diminished under conditions of severe injury. Using a targeted microarray approach focused on inflammatory genes, the study reveals that only immunization with A91 significantly modulates the expression of a subset of inflammation-related genes, with limited efficacy observed for Cop-1.

The *Bmp2 gene* encodes a bone morphogenetic protein (BMP) involved in neural repair and astrocytic reactivity,[20,21] *Bmp2* was significantly downregulated in the A91 group. This contrasts with the sustained expression in PBS and Cop-1 groups and may suggest that A91 helps attenuate astrocyte-driven gliosis, a major impediment to axonal regeneration in SCI. [20,22] Interestingly, while *Bmp3 gene*, a known antagonist of canonical BMP signaling,[23] was overexpressed in all groups, its functional role in SCI remains undefined. This divergence highlights the nuanced regulation of the BMP pathway, where the balance between members (e.g., *Bmp2* vs. *Bmp3*) may influence lesion architecture and the potential for regeneration.[20,21]

One of the most striking findings was the significant downregulation of *Ccl2*, *Casp1*, and *Casp3* genes exclusively in the A91 group. *Ccl2 gene encodes for Ccl2 protein that* is a chemokine critical for monocyte and T-cell recruitment.[24] Its reduction could limit excessive immune infiltration and consequent secondary tissue damage. Fang et al.[25] reported that *Ccl2* modulates the PI3K/Akt pathway, impacting apoptosis through regulation of *Bax* and *Caspase-3.* Thus, the concurrent downregulation of *Caspase-3* (apoptosis marker) and *Caspase- 1* (pyroptosis mediator) further supports the hypothesis that A91 immunization shifts the post-injury environment toward neuroprotection by dampening cell death mechanisms.[25,26] Pyroptosis, a highly inflammatory form of programmed cell death mediated via the NLRP3 inflammasome and Caspase-1,[26,27] is known to exacerbate SCI. A91-suppression of *Casp1 gene*, along with *Cebpb gene* and *Il1rap gene*—both involved in inflammasome signaling—suggests it may partially inhibit inflammasome activation, reducing pyroptosis and preserving neural tissue.[28]

Furthermore, the study found significant suppression of *SOCS genes* (Suppressor of Cytokine Signaling) family —*Cish*,

https://doi.org/10.36105/psrua.2025v5n9.01

Socs2, and *Socs3*— only in the A91 group. While SOCS proteins typically act as negative feedback regulators of cytokine signaling,[29,30] *Socs3* has been implicated in excitotoxic neuronal death and inflammation-induced injury in the CNS.[31,32] Its reduction may reflect a compensatory mechanism or could result in enhanced activation of anti-inflammatory signaling via STAT3, which has shown neuroprotective roles.[31] Similarly, the reduction in *Tgfb3 gene*, that encode a TGF-β superfamily member regulating Th17/Th1 balance, may indicate suppressed neuroinflammatory polarization.[33] The reduction in *Tnfsf11 gene* (RANK), which influences dendritic cell survival and immune activation via non-canonical NF-κB pathways,[34] suggests broader immunomodulatory effects mediated by A91.

Importantly, although A91 significantly decreased the expression of several key genes involved in immune modulation, apoptosis, and chemotaxis, these molecular effects did not translate into evident neuroprotection or improved outcomes in the context of severe SCI, as shown in prior work by our group.[7,19] This discrepancy may be attributed to the highly deleterious microenvironment generated after severe injury, particularly in the more advanced stages of the lesion. Such a hostile environment, characterized by sustained oxidative stress, pro-inflammatory cytokine release, and cellular necrosis, could override the potential benefits of A91-induced gene modulation. Therefore, despite its promising immunomodulatory profile, A91's clinical efficacy appears limited under severe injury conditions. This disconnection between gene expression changes and functional outcomes highlights a critical area for future research. Investigating the temporal evolution of the post-injury microenvironment and its impact on therapeutic responsiveness will be essential to optimize the timing and context of peptide-based interventions.

Interestingly, while *Il1b*, *Il18*, and *Tnf genes* showed numerical decreases in the A91 and Cop-1 groups, the changes were not statistically significant. These cytokines are central to SCI pathology, and their persistent expression might explain the limited functional recovery observed in prior severe SCI studies using these peptides.[7,19] The lack of suppression in other chemokines genes, such as *Ccl20* and *Ccl22*, across all groups suggests that certain inflammatory circuits remain unresponsive to peptide immunization, possibly due to overwhelming injury-related damage-associated molecular patterns (DAMPs) that continuously activate NF-κB and MAPK pathways.[35,36]

Despite the similarities between A91 and Cop-1 in immune modulation goals.[3,12,15] their divergent gene regulation patterns observed here reinforce that even subtle biochemical differences can drastically alter immunological outcomes. For instance, Cop-1 failed to significantly downregulate any of the eleven key inflammatory genes modulated by A91, consistent with previous findings that A91 is more effective in moderate SCI, but less so in severe models.[7,19]

These results suggest that the neuroprotective potential of peptide immunization is not solely dependent on peptide structure, but also on the severity of the injury and the existing inflammatory milieu. In cases of severe SCI, where immune activation and tissue damage are more profound, the ability of immunomodulatory therapies to tip the balance toward repair is diminished. Therefore, optimizing the timing, dosage, and possibly combining A91 with other anti-inflammatory or neuroregenerative agents may be necessary to overcome this limitation.

CONCLUSION

The present study provides compelling evidence that A91, in comparison with Cop-1, induces a focused anti-inflammatory gene expression profile in severe SCI, particularly affecting genes associated with apoptosis (Caspase-3), pyroptosis (Caspase-1, Cebpb), chemotaxis (Ccl2), and key signaling regulators (SOCS3, Tgfb3). However, the beneficial effects of A91 on gene expression are not reflected in functional outcomes after severe SCI, likely due to the noxious microenvironment generated in later stages of injury. It is imperative that future studies prioritize the investigation of the way this environment interferes with therapeutic interventions. Further research is required to evaluate the following:

- Dose-dependent responses.
- Time-course gene expression dynamics.
- The correlation between molecular changes and histological and behavioral outcomes Elucidation of these relationships may facilitate refinement and optimization of therapeutic immunization strategies in the context of SCI.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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