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Neuroimmune Crosstalk in Chicken Spleen Following H5N1 Vaccination: Serotonergic Network Analysis of Key Immune Genes

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Abstract

The spleen is a principal immune organ in poultry, but the molecular pathways mediating its response to avian influenza vaccination are poorly defined. This study aimed to explore the engagement of serotonergic system–related genes in the chicken spleen following vaccination with an H5N1 DNA vaccine (pCI-H5). Public microarray datasets (GSE135671 and GSE102972) were compared between vaccinated and control birds (receiving empty pCI plasmid) using GEO2R for differential gene expression; enriched pathways and protein–protein interaction networks were analyzed via STRING and Cytoscape. Fourteen genes were significantly differentially expressed post-vaccination, including CSF3R, LYG2, SERPINB10, DEFB4A, IFI6, and LECT2, which are involved in immune cell recruitment, inflammation modulation, apoptosis, and intercellular signalling. Enrichment analyses implicated pathways such as CCR6 chemokine receptor binding, beta-defensin activity, cellular chemotaxis, pathogen defence, and interleukin signalling. Interactions between serotonergic components and immune network nodes suggest a possible regulatory cross-talk. The serotonergic system may act as a modulatory hub in splenic immune activation following H5N1 DNA vaccination, and these serotonergic genes could serve as molecular targets to optimize future vaccine design and improve immune resilience in poultry.

Introduction

The spleen plays a pivotal role in avian immunity as a primary lymphoid organ responsible for initiating and regulating immune responses. It supports the maturation and proliferation of immune cells, particularly lymphocytes, which defend the host against infectious agents. Because of its central anatomical position, the spleen serves as a key indicator of systemic immune activation following vaccination, and is therefore frequently examined in immunological studies to assess both vaccine responsiveness and overall immune status in birds (Lewis *et al.*, 2019; Zhang *et al.*, 2019). Structurally, the chicken spleen consists of two main compartments: red pulp and white pulp (Hamza, 2017; Nnadozie *et al.*, 2025). The red pulp is primarily involved in blood filtration and the clearance of aged or damaged erythrocytes, whereas the white pulp mediates lymphocyte activation and proliferation (Abdulloyevich *et al.*, 2023). In contrast to mammals, chickens lack organized lymph nodes and rely instead on diffuse lymphoid tissues scattered throughout their organs to perform comparable immunological functions (Lewis *et al.*, 2019).

The serotonergic system, one of the most significant monoaminergic pathways, governs multiple physiological processes including mood, feeding behaviour and cognition. Importantly, serotonin (5- hydroxytryptamine; 5- HT) acts not only as a neurotransmitter but also as an immunomodulatory molecule in peripheral tissues. It operates across both the central and peripheral nervous systems. Serotonin synthesis involves a two- step enzymatic process starting from tryptophan, catalysed by tryptophan hydroxylase and aromatic L- amino acid decarboxylase (Berger *et al.*, 2009). Recent research

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has also uncovered bidirectional communication between serotonergic and immune pathways, whereby immune activation can modify serotonin levels and, conversely, serotonergic signalling can influence immune gene expression and cytokine release (Lyte *et al*., 2025). This emerging concept of neuroimmune crosstalk provides novel insight into how neural mediators can shape immune responses during vaccination.

Highly pathogenic avian influenza virus (HPAI) subtype H5N1 remains a critical global threat to poultry health, causing recurrent outbreaks and considerable economic losses (Niu *et al*., 2025). Owing to its zoonotic potential, occasional human infections result in severe respiratory disease and high mortality rates (Charostad *et al*., 2023). Continuous surveillance, robust biosecurity, and effective vaccination remain key strategies for its control. Beyond conventional vaccine evaluation, understanding the molecular pathways linking neuroendocrine and immune responses could reveal crucial determinants of vaccine efficiency in poultry.

Therefore, the present study aimed to explore immune gene expression patterns potentially associated with serotonergic neuroimmune interactions in the chicken spleen following repeated H5N1 DNA vaccination using integrated microarray analyses of public datasets (GSE135671 and GSE102972) that utilized an H5-expressing DNA construct. This approach provides insight into how neuroimmune signaling potentially linked to the serotonergic system, may participate in splenic immune regulation and identifies hub genes that may serve as molecular targets for enhancing DNA vaccine-induced immunity in avian species.

Materials and Methods

Data Source and Preprocessing

Accessed GSE135671 and GSE102972 microarray datasets were acquired from the Gene Expression Omnibus (GEO) databases of the National Center for Biotechnology Information (NCBI) and were produced using the Affymetrix chicken genome array (GPL3213). Chickens were immunized twice via intramuscular injection with a 21-day (three-week) interval between the primary dose and the booster. The vaccine was a DNA vaccine (pCI-H5) encoding the codon-optimized H5 hemagglutinin (HA) gene derived from the A/swan/Poland/305-135V08/2006 (H5N1) strain, which was cloned into the pCI expression vector (Promega). The control group received the empty pCI plasmid under identical conditions. For the microarray analysis, spleen tissues were harvested on Day 28 (exactly 7 days after the booster immunization) to capture the peak vaccine-induced transcriptomic response (Kalenik *et al*., 2020). A total of 10 samples were integrated from two datasets for this analysis: GSE135671, providing three samples from DNA-vaccinated chickens (GSM4025703–GSM4025705) and three samples from empty-vector controls (GSM4025706–GSM4025708); and GSE102972, providing two samples from DNA-vaccinated chickens (GSM2752000–GSM2752001) and two samples from empty-vector controls (GSM2751998–GSM2751999) (Table 1). GEO2R (ver. as of 2024) (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was used to carry out preprocessing as well as data differential expression analysis. For the CEL files, the quality control procedures were carried out to ensure that files from the different datasets were comparable across the datasets. For the identification of differentially expressed genes, $\log_2$ fold change ($|\log FC| \geq 1.0$) and adjusted *P*-value (FDR< 0.05) were used. The expression matrices of the datasets were merged by gene symbol, and only shared probes were retained for analysis. The genes that met the specified statistical criteria were exported to Excel for downstream analysis.

Functional Enrichment Analysis

Functional annotation and enrichment analyses were carried out to explore the biological roles of significantly expressed genes. Gene Ontology (GO) term enrichment (biological process, molecular function, and cellular component) and Reactome pathway analyses were conducted using the STRING database (version 12.0). Pathway enrichment was considered statistically significant at FDR< 0.05. In STRING, the minimum required interaction score was set to 0.7 (high confidence), and the organism parameter was restricted to *Gallus gallus*.

Network Visualization and Topological Analysis

A protein–protein interaction (PPI) network was constructed in STRING (v12.0) and subsequently imported into Cytoscape (v3.10.0) for visualization and topological analysis. Disconnected nodes were removed to improve network clarity. Network centrality metrics, including degree, betweenness, and closeness centrality, were computed using the CytoNCA plugin (v2.1.6) with normalization enabled. Hub genes identified based on these centrality parameters were further interpreted within the context of serotonergic–immune cross-regulation in the chicken spleen.

Table 1: GEO accession numbers of spleen samples from chickens immunized with pCI-H5 DNA vaccine or empty vector control.

Access number	pCI-H5 DNA vaccine	Empty pCI vector control
GSE135671	GSM4025703	GSM4025706
	GSM4025704	GSM4025707
	GSM4025705	GSM4025708
GSE102972	GSM2752000	GSM2751998
	GSM2752001	GSM2751999

Results

Differential expression was observed in 14 genes, of which two were downregulated, and twelve were upregulated (Table 2). The cross-integration of the two microarray datasets (GSE135671 and GSE102972), both normalized using the RMA algorithm, ensured that the observed expression changes reflected biological variation rather than technical noise. Thus, these 14 genes represented consistent differential expression patterns across vaccinated and control groups, providing a reliable basis for subsequent functional annotation and pathway analysis. Functional enrichment analysis was performed using the STRING database, incorporating Gene Ontology (GO) and Reactome annotations at a false discovery rate (FDR < 0.05). In the GO Biological Process category, the most significantly enriched terms included cell chemotaxis, defence response to bacterium, response to bacterium, defence response to other organism, and response to external stimulus. In the GO Molecular Function category, enriched terms included CCR6 chemokine receptor binding and chemoattractant activity. Reactome pathway analysis further revealed enrichment of beta-defensin–related pathways and interleukin-associated signalling processes (Figures 1–3). Together, these findings suggest that the vaccination was associated with activation of innate immune–related and cytokine-mediated processes in the spleen. To examine the interaction landscape among these differentially expressed genes, a protein–protein interaction (PPI) network was generated using the STRING database and visualized in Cytoscape (Figure 4). Network topology analysis based on degree, betweenness, and closeness centrality measures identified six highly connected genes, CSF3R, LYG2, SERPINB10, DEFB4A, IFI6, and LECT2, which may represent important nodes within the splenic immune interaction network following H5N1 DNA vaccination (Table 3).

Table 2: Differentially expressed genes identified in chicken spleen tissue following immunization with the pCI-H5 DNA vaccine.

Gene names	*P*-value	log2 Fold Change (Vaccinated vs. Empty vector) *	Regulation
TPD52L1	0.0166	-1.21	Downregulated
IFNLR1	0.0005	-1.01	Downregulated
YG2	0.0243	1.01	Upregulated
CSF3R	0.0098	1.14	Upregulated
IFI6	0.0025	1.15	Upregulated
ACOD1	0.0286	1.15	Upregulated
GVINP1	0.0005	1.21	Upregulated
MX1	0.0257	1.31	Upregulated
SERPINB10	0.0002	1.33	Upregulated
AvBD6	0.0004	1.66	Upregulated
LECT2	0.0054	1.7	Upregulated
AvBD1	0.0002	2.16	Upregulated
DEFB4A	0.0047	2.23	Upregulated
AvBD7	0.0092	2.29	Upregulated

***** Fold-change values represent gene expression levels in chickens vaccinated with the pCI-H5 DNA vaccine relative to chickens immunized with the empty pCI vector control.

Our findings suggest that the immune gene expression changes observed after repeated H5N1 vaccination may be indirectly associated with broader neuroimmune regulatory processes, potentially including serotonergic pathways. Among the identified genes, CSF3R and DEFB4A were of particular interest because of their central positions within the interaction network, indicating potential roles as integrative nodes within immune signalling frameworks that may interface with neuroendocrine regulatory mechanisms. Together with the immune genes LYG2, SERPINB10, IFI6, and LECT2, these molecules outline a possible neuroimmune context in chickens in which immune signalling pathways may interact with neuroendocrine mediators during the vaccine-induced splenic response. Overall, this work suggests that the interconnected gene networks comprising CSF3R, LYG2, DEFB4A, SERPINB10,

IFI6, and LECT2 may represent components of a broader neuroimmune interface, potentially reflecting indirect interactions between immune signalling pathways and serotonergic modulation.

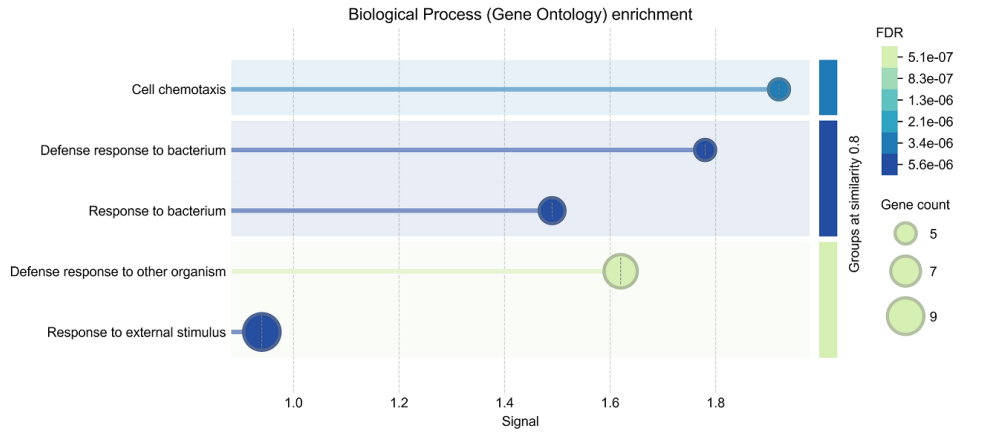


Figure 1. Gene Ontology (GO) enrichment analysis of Biological Processes associated with differentially expressed genes in the chicken spleen following H5N1 DNA vaccination.

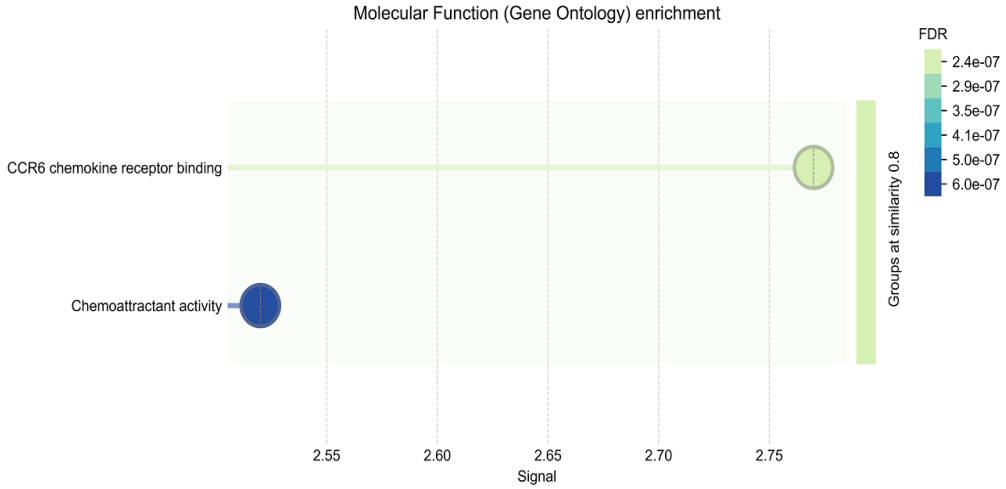


Figure 2. Gene Ontology (GO) enrichment analysis of Molecular Functions of differentially expressed genes identified after H5N1 DNA vaccination.

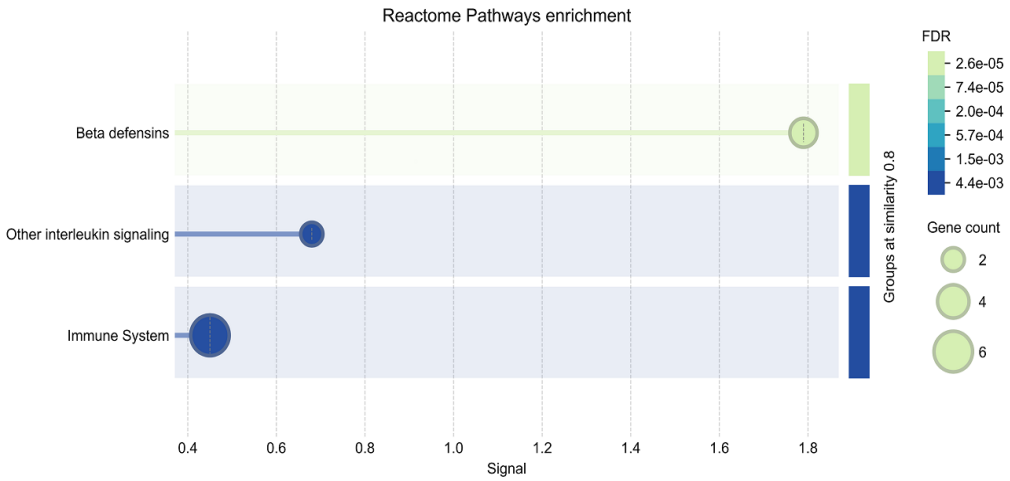


Figure 3. Reactome pathway enrichment analysis of differentially expressed genes in the chicken spleen following H5N1 DNA vaccination.

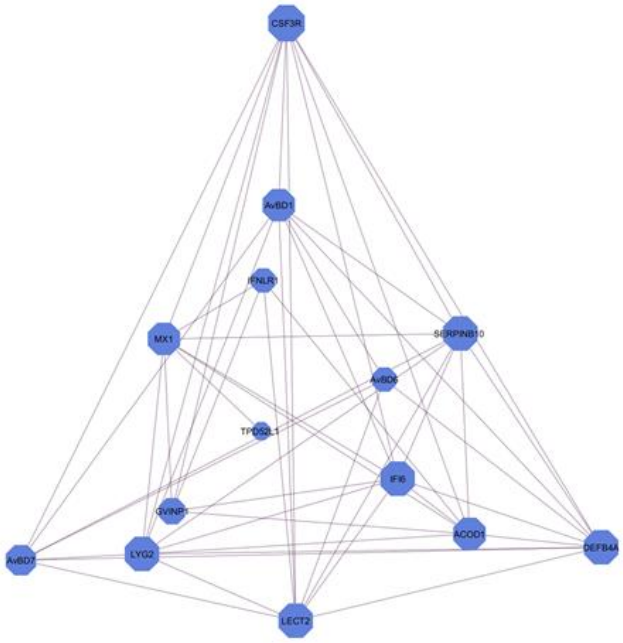


Figure 4. Protein–protein interaction network of differentially expressed genes identified in the chicken spleen following H5N1 DNA vaccination. Interaction data were obtained from the STRING database (version 12.0) and visualized in Cytoscape (version 3.9.1). Nodes represent proteins corresponding to differentially expressed genes, and edges show predicted functional associations.

Table 3: Network topological parameters (degree, betweenness, and closeness centrality) of differentially expressed genes identified in the chicken spleen following H5N1 DNA vaccination. Degree centrality reflects the number of direct connections of each gene in the network, betweenness centrality reflects its role as a potential hub or mediator on shortest paths between other genes, and closeness centrality reflects the efficiency of information flow from that gene to all other genes in the network.

Gene name	Degree	Betweenness	Closeness
CSF3R	10	12.663399	0.8125
LYG2	9	6.4411764	0.7647059
SERPINB10	9	6.4411764	0.7647059
DEFB4A	9	7.5764704	0.7222222
IFI6	9	8.039216	0.7647059
LECT2	9	13.676471	0.7222222

Discussion

This study analyzed splenic transcriptional responses following consecutive H5N1 vaccinations to identify immune-relevant genes and to explore their potential relationships with serotonergic-associated signalling in the chicken spleen. Overall, repeated vaccination was accompanied by significant modulation of immune responses and concomitant activation of key immune genes, including SERPINB10, DEFB4A, IFI6, and LECT2, alongside central network nodes such as CSF3R and LYG2. These changes suggest that successive H5N1 immunization triggers a coordinated program in the spleen that integrates innate effector activation, interferon-driven antiviral defence, chemotactic recruitment, and immune cell survival, all of which are critical for shaping vaccine responsiveness.

Beyond classical immune pathways, the chicken serotonergic system is increasingly recognized as a modulator of peripheral immunity, including splenic immune functions (De Haas & van der Eijk, 2018). Serotonin (5-HT) can act as an immune-modulating neurochemical and may influence leukocyte activity and inflammatory balance (Jadhav *et al*., 2022; Linh *et al*., 2021). In the context of repeated vaccination, bidirectional neuroimmune communication has been proposed, where immune activation may alter serotonin homeostasis and serotonergic signalling may, in turn, fine-tune immune outputs (Lyte *et al*., 2025). Consistent with this concept, our network-level results support the hypothesis that H5N1 vaccine-induced immune activation in the spleen may intersect with broader serotonergic-related regulatory layers, even if the differentially expressed genes are not classical serotonergic receptors.

A prominent hub in our PPI-derived network was CSF3R, a key regulator of granulocyte biology. CSF3R signalling is central to granulocyte

development, differentiation, survival, and chemotaxis functions that are directly relevant to rapid innate responses after vaccination (Dwivedi & Greis, 2017; Park *et al*., 2022; Lance *et al*., 2024). Through binding of G-CSF, CSF3R activates major downstream pathways, notably JAK/STAT, MAPK, and PI3K, which collectively support expansion and functional mobilization of granulocytic lineages (Dwivedi & Greis, 2017; Park *et al*., 2022; Lance *et al*., 2024). In birds, heterophils represent functional counterparts to mammalian neutrophils; thus, the post-vaccination modulation of CSF3R is compatible with enhanced innate readiness in the spleen, potentially improving early containment mechanisms against influenza challenge. Moreover, CSF3R-mediated granulocyte activity is likely coordinated with cytokine networks, including interleukin-linked signalling modules, suggesting synergistic amplification of inflammatory and antimicrobial functions rather than isolated signaling (Leiba *et al*., 2023). While direct serotonergic transcriptional regulation of CSF3R is not well established in avian species, shared use of downstream signalling axes and the network centrality of CSF3R support its potential placement within a broader neuroimmune interface activated after H5N1 vaccination.

Another gene highlighted by our analysis was LYG2, which has been associated with enhanced antimicrobial defence and immune activation during infectious or vaccine-driven immune stimulation (Zhang *et al*., 2021). Increased LYG2 expression following immune challenge supports its role in strengthening innate effector capacity, and available evidence suggests involvement in immune-cell recruitment and migration processes that can shape the local splenic microenvironment post-vaccination (Hughes & Nibbs, 2018; Meijer *et al*., 2025). Importantly, recent work has suggested that vaccination-associated immune activity can be linked to serotonergic homeostasis and that serotonergic pathways may participate in orchestrating immune modulation in poultry (Lyte *et al*., 2025; Meijer *et al*., 2025). In this framework, LYG2 may represent a splenic innate effector component whose expression reflects both vaccine-induced immune activation and broader regulatory inputs that include neuroimmune signalling.

Among the most consistently immune-relevant candidates, SERPINB10 represents a serine protease inhibitor implicated in immune regulation and apoptosis control. Reactome-based annotations place SERPINB10 within immune system pathways, with an emphasis on regulating cell survival during immune activation (Vastrad & Vastrad, 2023). Following repeated H5N1 vaccination, maintenance of immune effector cell viability is essential for sustaining antigen processing and downstream responses; thus, SERPINB10 upregulation is biologically consistent with an activated yet controlled splenic response. In addition, given evidence for bidirectional serotonin–immune interactions during vaccination-driven activation (Lyte *et al*., 2025), SERPINB10 may participate in immune-state stabilization that indirectly interfaces with neuroimmune modulation, though mechanistic validation remains necessary.

DEFB4A, a beta-defensin, supports the first line of defence via direct antimicrobial activity and broader immunoregulatory roles, including leukocyte-associated immunity and myeloid differentiation (Wu *et al*., 2020). Beta-defensins may also act as chemoattractants and shape leukocyte recruitment, including through CCR6-related migratory signalling (Caballero- Campo *et al*., 2014). Such functions are particularly relevant in the spleen after consecutive H5N1 vaccinations, where effective immune-cell trafficking and rapid effector deployment can influence vaccine responsiveness. Moreover, defensins have been implicated as enhancers of vaccine-induced immunity, including amplification of antiviral cytokine production and strengthening of both humoral and cellular arms of immunity (Kawada-Matsuo & Komatsuzawa, 2017). Therefore, the differential expression of DEFB4A in our data aligns with a spleen-centered enhancement of innate and downstream adaptive-supportive responses following repeated H5N1 immunization.

The interferon-stimulated gene IFI6 is among early-responsive ISGs during viral infection and has been shown to increase markedly under challenges such as highly pathogenic avian influenza and other viral infections (Wan *et al*., 2023). IFI6 expression across key lymphoid tissues, including the spleen, supports its role in orchestrating early antiviral defence and cell survival programs during immune activation (Wan *et al*., 2023). In the setting of repeated H5N1 vaccinations, modulation of IFI6 is consistent with reinforcement of interferon-mediated antiviral readiness, which is central to protective responses against influenza-like threats.

Finally, LECT2 has been associated with chemotactic and immunoregulatory functions, including supporting immune activation and shaping inflammatory balance. Recent work suggests LECT2 can augment antiviral defences by engaging innate viral-sensing pathways such as RIG-I signalling (Zhu *et al*., 2023). Given that influenza virus sensing and early antiviral signalling are crucial determinants of protective immunity, LECT2 upregulation after consecutive H5N1 vaccinations may indicate strengthened innate antiviral responsiveness in the spleen, potentially contributing to improved vaccine-mediated preparedness.

Taken together, our results highlight that consecutive H5N1 vaccination is accompanied by coordinated splenic immune activation involving granulocyte-associated regulation (CSF3R), antimicrobial effectors (LYG2, DEFB4A), interferon-driven

antiviral programs (IFI6), immune survival regulation (SERPINB10), and chemotactic/antiviral modulators (LECT2). In parallel, the network-level association with serotonergic-related regulation supports a model in which serotonin acts as a contextual immunomodulator rather than a direct immune checkpoint in the spleen after vaccination (de Haas & van der Eijk, 2018; Jadhav *et al*., 2022; Lyte *et al*., 2025). Future in vivo studies targeting serotonin receptors or serotonin availability, alongside immunophenotyping of splenic leukocyte subsets, would be valuable to empirically validate the proposed neuroimmune crosstalk and to determine whether serotonergic modulation can be leveraged to optimize H5N1 vaccine responses in poultry.

Conclusion

The findings of this study provide additional insight into the potential interaction between serotonergic signalling and immune pathways in the chicken spleen following H5N1 DNA vaccination. By integrating publicly available microarray datasets, several differentially expressed genes, including CSF3R, LYG2, SERPINB10, DEFB4A, IFI6, and LECT2, were identified as components of a small immune-related molecular network associated with post-vaccination responses. These genes are functionally linked to processes such as immune cell recruitment, antimicrobial activity, antiviral defence, and inflammatory regulation. The network analysis suggests that serotonergic signalling pathways may interact with immune regulatory mechanisms in the spleen, indicating a possible neuroimmune crosstalk during the vaccination response. Although the present analysis does not establish direct causal relationships, it highlights candidate genes and pathways that could contribute to immune modulation following vaccination. Overall, these findings provide a systems-level perspective on splenic immune responses to H5N1 DNA vaccination and may support future studies aimed at improving vaccine efficacy and immune resilience in poultry through a better understanding of neuroimmune interactions.

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Authors' Contribution

Conceptualization, Z.R. and F.M.; Methodology, Z.R. and F.M.; Software, Z.R. and F.M.; Validation, Z.R., F.M., and Y.B.; Formal analysis, Z.R. and F.M.; Investigation, Z.R. and Y.B.; Resources, Z.R. and F.M.; Writing – original draft preparation, F.M.; Writing – review and editing, Z.R. and Y.B.; Visualization, Z.R. and F.M.; Supervision, Z.R.; Project administration, Z.R.; Data acquisition, Z.R. and Y.B.; Data interpretation, Z.R. and F.M.; Critical revision for important intellectual content, Z.R. and Y.B.; Statistical analysis, Z.R. and F.M.; Technical and material support, Z.R. All authors have read and agreed to the published version of the manuscript.

Ethical Statement

In this study, we analyzed data sets freely available in the public domain. Accordingly, ethics committee approval was not required.

Conflict of Interest

The authors declare that they have no conflict of interest related to this study.

Data Availability

All data analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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