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***Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* exert anti-inflammatory effects through distinct immunomodulatory mechanisms in murine macrophages *in vitro*.**

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## **Abstract**

Hookworm infections affect hundreds of millions of people worldwide and rank among the most significant neglected tropical diseases in terms of morbidity. Due to the inverse correlation between hookworm infection and the incidence of immune-mediated inflammatory diseases, considerable research has focused on understanding how parasitic helminths modulate host inflammation. *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* are two hookworm-like rodent models widely used to investigate fundamental aspects of immune regulation. However, no comparative study has yet analysed the distinct immunological pathways activated by these helminths in their hosts. In this study, we compared cytokine profiles and M1/M2 macrophage polarization markers induced by *H. polygyrus* and *N. brasiliensis*, alongside

proteomic pathways involved in their activation, using the RAW 264.7 murine macrophage cell line. *H. polygyrus* downregulated proinflammatory cytokines such as TNF- $\alpha$  and MCP-1, whereas *N. brasiliensis* did not affect TNF- $\alpha$  expression. Both parasites upregulated Th2 and regulatory cytokines, including IL-4 and TGF- $\beta$ . Furthermore, *H. polygyrus* predominantly polarized macrophages toward an M2 phenotype, while *N. brasiliensis* maintained a balanced M1/M2 profile. Proteomic analysis revealed that *N. brasiliensis* and *H. polygyrus* exert their anti-inflammatory effects through distinct mechanisms, with *N. brasiliensis* acting via peripheral pathways and *H. polygyrus* via central regulatory mechanisms.

**Keywords:** *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus*, RAW264.7, macrophages, nematoda, immunomodulation

## 1 INTRODUCTION

Infections caused by parasitic helminths have a high prevalence worldwide, in particular in non-industrialised countries from tropical and subtropical regions [1,2]. According to the WHO, in 2018, 25% of the global population was infected with a helminth species, with nematodes being the most predominant group [3]. More importantly, these infections are associated with significant morbidity and mortality, particularly in children [4]. Species from the genera *Ascaris*, *Ancylostoma*, *Necator*, *Strongyloides*, and *Trichuris*, all known to parasitize their hosts through soil contact, infect approximately one billion people worldwide, significantly contributing to disability-adjusted life years (DALYs) particularly in impoverished and resource-limited settings. [2].

Despite the negative effects associated with parasites, there is evidence that their complete eradication can have adverse consequences for human health. Indeed, it is well known that limited exposure to pathogens leads to an underdeveloped regulatory immune system, ultimately increasing the prevalence of immune-related disorders [5,6]. Nematodes, aiming to survive within their host, must evade or modulate the immune response in their favour, strongly down-regulating host inflammatory responses [6]. This immunomodulatory ability affects the immune response in various ways, such as inhibiting protective mechanisms like mast cell degranulation, impairing the induction of immune responses, and promoting regulatory T cell induction [7]. These mechanisms

are primarily mediated by bioactive molecules excreted and/or secreted by the parasites known as excretory/secretory products (ESPs) [6,8].

Hookworms have been described as one of the groups of nematodes with the highest immunomodulatory potential [9], and some of their secreted molecules ameliorate the symptoms of inflammatory diseases such as asthma and inflammatory bowel disease [6,10]. Due to the difficulty in reproducing human hookworms' life cycle in laboratory settings, different hookworm-like rodent nematodes such as *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* have been widely used for the study of hookworm biological features. Indeed, recent proteomic and transcriptomic studies have shown evidence of the high similarity between these rodent models and their human counterparts, making them highly suitable for the study of the immunobiology of gastrointestinal nematode infections [11].

*N. brasiliensis* belongs to the phylum Nematoda and the superfamily Trichostrongyloidea, with rats as its natural hosts, though it can also infect other rodent species like mice. Primary infection elicits pulmonary protection against reinfection, which depends on CD4<sup>+</sup> T cells and alternatively activated macrophages (M2). These M2 macrophages may also contribute to tissue repair following the lung phase of the parasite's life cycle [12]. In mice, infection with this parasite triggers a dominant Th2 response characterized by increased IL-4, IL-5, and IL-13 cytokines and elevated IgE antibody production [13]. Numerous studies highlight *N. brasiliensis* as a potent immunomodulator, with ESPs playing a significant role in this process [14,15]. Among these ESPs, proteins belonging to the SCP/TAPS family stand out as key mediators in evasion of the host's immune response [15].

The gastrointestinal nematode *Heligmosomoides polygyrus* belongs to the phylum Nematoda, superfamily Trichostrongyloidea, and family *Heligmosomidae*, with wild house mice (*Mus musculus*) as its natural host. Despite the similarities at a proteomic and transcriptomic level with human hookworms, *H. polygyrus* has a slightly different life cycle. In the case of *H. polygyrus*, definitive hosts acquire infection by oral ingestion of infective larvae, which then invade the duodenal mucosa, penetrating the muscular layer to reside beneath the serosal membrane, and returning to the intestinal lumen as adult worms about eight days later [16]. Due to its manageable life cycle in the laboratory, *H. polygyrus* has been widely used to study immunomodulation [17]. Infection is accompanied by regulatory T cell populations, dendritic cells, macrophages, B cell hyperstimulation, and localized changes in the intestinal environment [17]. *H. polygyrus* ESPs

include HpARI (*H. polygyrus* Alarmin Release Inhibitor), which suppresses the release of alarmins like IL-25 and IL-33 produced by epithelial cells [18,19]. More recently, a TGF- $\beta$  mimic molecule secreted by *H. polygyrus* has been identified to activate TGF- $\beta$  signalling and inducing the proliferation of mouse and human Foxp3<sup>+</sup> Treg cells [20]. Additionally, *H. polygyrus* secretions contain apyrases that degrade ATP, reducing inflammatory DAMP signals and inhibiting damage detection responses [21].

Although *N. brasiliensis* and *H. polygyrus* differ in their life cycles, tissue tropism, and immunomodulatory strategies, macrophages emerge as a central cellular component shaping host responses to both parasites [22]. Helminth ESPs represent a key interface through which parasites directly modulate macrophage signalling pathways, influencing downstream adaptive immune responses [23]. As such, macrophages provide a unifying framework for integrating helminth-specific immunomodulatory mechanisms and represent an ideal cellular system in which to compare host signalling pathways activated by distinct helminth species [22].

Despite recent efforts in studying the bioactive molecules from *N. brasiliensis* and *H. polygyrus* ESPs, to our knowledge, no comparative study analysing the different pathways activated by both helminths in their hosts have been performed. Thus, the main objective of this work is to identify the key signalling pathways activated by both helminths in murine macrophages. This study provides insights into the similarities and differences in host-pathogen interactions mediated by two of the most widely used helminths in immunomodulatory studies. Given the growing interest in helminth-derived molecules as candidate therapeutics for TLR4-driven inflammatory conditions such as inflammatory bowel disease, we designed this study as a pharmacological screening approach to understand the mechanisms underlying the anti-inflammatory potential of nematode ESPs. RAW264.7 macrophages were challenged with a standardised, well-characterised pro-inflammatory stimulus (LPS), and the capacity of NbESP and HpESP to suppress this established inflammatory response was benchmarked against dexamethasone, a gold-standard reference anti-inflammatory drug. This framework, rather than attempting to reproduce the Th2/alarmin-driven microenvironment of natural helminth infection, allowed us to directly and comparatively assess the mechanisms underlying the anti-inflammatory effects of both ESPs.

## 2 RESULTS

## 2.1 *H. polygyrus* ESPs exert a more potent anti-inflammatory effect on murine macrophages *in vitro* compared to *N. brasiliensis* ESPs following LPS stimulation.

To comparatively assess the immunomodulatory properties of helminth-derived ESPs, we investigated the ability of *H. polygyrus* (*HpESP*) and *N. brasiliensis* (*NbESP*) to modulate the inflammatory response of murine macrophages following LPS stimulation. Cytometric analyses enabled the quantification of five cytokines in total. Standard curves were generated for each cytokine, yielding coefficients of determination ( $R^2 \geq 0.99$ ). Upon interpolation of the signal obtained for each cytokine on the corresponding standard curve, the resulting concentrations are presented in Figure 1, which also highlights the statistically significant differences identified through subsequent statistical analyses.

*HpESP* was able to reverse the previously LPS-induced stimulation of TNF- $\alpha$  (Figure 1A). Significant differences were observed in the groups treated with LPS in combination with the anti-inflammatory corticosteroid dexamethasone (LPS + Dx) ( $p < 0.005$ ) or *HpESP* ( $p < 0.001$ ), compared to LPS-treated control. However, no statistically significant changes were detected in the *NbESP* + LPS group (Figure 1A). These findings were further validated by ELISA, confirming the results obtained by CBA. As observed in the CBA assay, treatment with LPS + Dx significantly reduced TNF- $\alpha$  levels ( $p < 0.01$ ) compared to the LPS-stimulated group (Supplementary Figure 1). Similarly, a significant reduction ( $p < 0.01$ ) was observed in the *HpESP* + LPS group. No statistically significant differences were detected in the *NbESP* + LPS group.

Interestingly, in the case of the monocyte chemoattractant protein-1 (MCP-1), a statistically significant reduction was observed for *HpESP* + LPS ( $p < 0.001$ ), *NbESP* + LPS ( $p < 0.05$ ), and LPS + Dx ( $p < 0.05$ ) (Figure 1B). Regarding the concentration levels of IL-6, significant differences were observed after treatment with either Dx, *HpESP* and *NbESP* compared to the LPS-only stimulated group ( $p < 0.01$ ) (Figure 1C). In this case, expression of IL-6 was reduced by 68.3%, 79.8%, and 92.7% following treatment with LPS + Dx, *NbESP* + LPS, and *HpESP* + LPS, respectively (Figure 1C). On the contrary, no significant differences were observed across experimental groups for IL-12p70 (Figure 1D) and IL-10 (Figure 1E).

The expression of TGF- $\beta$  and IL-4 genes was measured by qPCR. Interestingly the expression in both cases was significantly increased in RAW264.7 macrophages after treatment with *NbESP*+LPS and *HpESP*+LPS, although *NbESP* seemed to have a significantly higher effect on the expression of TGF- $\beta$  (Figure 1F, G). In the case of TGF- $\beta$ , expression increased 2,612-fold

and 1,791.5-fold following treatment with *Nb*ESP+LPS and *Hp*ESP+LPS, respectively, compared to the control (Figure 1F). Similarly, IL-4 expression increased 77.1-fold and 124.7-fold after treatment with *Nb*ESP+LPS and *Hp*ESP+LPS, respectively (Figure 1G).

## **2.2 *Nb*ESP triggers a broader concurrent induction of M1 and M2 associated markers than *Hp*ESP in RAW 264.7 macrophages**

Genes associated with macrophage polarization towards classical and alternative activation were analysed by qPCR. The expression of Ym1 and ARG, two genes positively implicated in the polarization towards a M2 phenotype of macrophages, was analysed. Treatment with *Nb*ESP + LPS and *Hp*ESP + LPS (as well as with the positive control LPS + Dx) significantly induced the expression of Ym1 (Figure 2A). In the case of *Nb*ESP + LPS the expression was increased 317.1 times ( $p < 0.0001$ ), while 76.1 times ( $p < 0.005$ ) when cells were treated with *Hp*ESP + LPS (Figure 2A). Similarly, the expression of ARG was significantly induced 207.2 ( $p < 0.0001$ ) and 65.3 times ( $p < 0.0005$ ) after treatment with *Nb*ESP + LPS and *Hp*ESP + LPS, respectively (Figure 2B).

In the case of iNOS, an enzyme implicated in polarization towards a M1 phenotype, treatment of cells with *Nb*ESP + LPS and *Hp*ESP + LPS significantly induced the expression of this gene compared to controls (Figure 2C;  $p < 0.05$ ). Interestingly, the expression of iNOS increased 180.5 times after treatment with *Nb*ESP + LPS, while only 20.1-fold when cells were treated with *Hp*ESP + LPS (Figure 2C).

To further assess whether *Nb*ESP and *Hp*ESP possess an intrinsic capacity to induce macrophage polarisation independently of an inflammatory trigger, we next evaluated the effect of each ESP alone (i.e., without LPS co-stimulation) on the expression of Ym1, ARG and iNOS, together with the corresponding untreated and LPS-alone controls (Supplementary Figure 2). *Nb*ESP alone was sufficient to significantly induce both M2-associated markers (Ym1 and ARG) and the M1-associated marker iNOS relative to untreated controls. In contrast, *Hp*ESP alone did not significantly induce Ym1 or ARG expression, and produced only a modest, though statistically significant, increase in iNOS. LPS alone did not significantly alter the expression of any of the three markers relative to control (Supplementary Figure 2).

## **2.3 *Hp*ESP and *Nb*ESP induce different proteomic expression profiles in content and amount of differentially expressed proteins**

For a more comprehensive analysis of the mechanisms underlying the anti-inflammatory potential of *H. polygyrus* and *N. brasiliensis*, RAW264.7 cells incubated with *Hp*ESP and *Nb*ESP were analyzed by LC-MS/MS. A total of 64,843 spectra were acquired and used to assign 49,121 unique peptides, leading to the identification of 4,348 proteins (4,281 protein groups). After removing *N. brasiliensis*, *H. polygyrus* and contaminant proteins, a total of 4,245 protein groups were kept for further analyses. The quantification analysis was performed using Spectronaut and only proteins with a *q* value <0.05 and a log<sub>2</sub> fold-change >0.58 or <-0.58 (for upregulated and downregulated proteins, respectively) were taken into consideration for further analysis. After removing potential contaminants and parasite proteins, a total of 990 and 2,222 proteins had significantly dysregulated expression profiles in RAW264.7 cells after treatment with *Hp*ESP and *Nb*ESP, respectively (Supplementary Table 1).

PCA was conducted to reduce data dimensionality and visualize sample clustering. The resulting PCA plot shows that the first two principal components, PC1 and PC2, explain 35.7% and 21.3% of the total variance, respectively. The samples cluster differently according to their treatment groups (Figure 3A), supporting that exposure to *Nb*ESP and *Hp*ESP potentially induces distinct proteomic profiles in RAW264.7 cells. This was further supported by the amount of differentially expressed proteins (DEPs) with respect to controls (Figure 3B, C).

#### **2.4 *N. brasiliensis* and *H. polygyrus* ESPs activate different inflammatory-related pathways in murine macrophages**

To better understand the pathways involved in immune modulation by *N. brasiliensis* and *H. polygyrus*, proteins with the GO term “Immune system response” (GO:0002376) as a parent term were further analysed. A protein-protein interaction network was generated using StringDB data and clusters were manually curated (Figure 4A). The network revealed a central cluster of 109 proteins primarily associated with common immune responses to helminths, including activation of IL-5 (Th2 response) and other cytokine signalling pathways.

In addition to this central node, several smaller clusters (comprising 2–4 proteins each, *e.g.*, Progranulin, Prosaposin, Protein unc-93 homolog B1, Toll-like receptor, ATPase family, Evolutionarily conserved signalling intermediate in Toll pathway, subunits of the AP-3 complex, Vesicle-associated membrane protein 7 (VAMP-7), MAP kinase-activated protein and Rab-27A) were identified and found to be directly involved in anti-inflammatory responses. Interestingly, most of the proteins involved in these “anti-inflammatory pathways” were directly regulated by *N.*

*brasiliensis*. However, two proteins related to lysosome metabolism (e.g. Progranulin, Prosaposin) and a protein involved in innate and adaptive immunity (Protein unc-93 homolog B1) were regulated by *H. polygyrus*. Other clusters were enriched in proteins involved in Th1-related biological processes such as nucleotide metabolism (e.g. Impdh1 and ctps1), mRNA regulation (RNA demethylase, YTH domain-containing family protein 2) proteasome-mediated antigen processing (Proteasome activator complex subunits), and T-cell receptor signalling (several subunits of the translational initiation factor 2B (eIF2B)).

To further investigate the functional relevance of these proteins, enrichment analyses were performed using STRINGdb. WikiPathways analysis of proteins annotated with the GO term “Immune system” and differentially regulated by *H. polygyrus* (Figure 4B) revealed enrichment of pathways associated with phagocytic innate immune responses, including the “Microglia pathogen phagocytosis” pathway (e.g. Trem2) together with pathways involved in oxidative stress responses and complement activation. In contrast, proteins differentially regulated by *N. brasiliensis* were enriched in cytokine-signalling pathways, including IL-5, IL-6, IL-3 and IL-7 signalling, as well as NF- $\kappa$ B-associated pathways (e.g. Stat3). Reactome analysis further highlighted distinct pathway signatures for each species (Supplementary Figure 3). Proteins regulated by *H. polygyrus* were enriched in pathways related to innate and humoral immune responses, including “Neutrophil degranulation”, “Classical antibody-mediated complement” and “Regulation of Complement Cascade” (Supplementary Figure 3A). In contrast, proteins differentially regulated by *N. brasiliensis* were enriched in cytokine- and interleukin-mediated signalling pathways, including “Signaling by Interleukins”, “Cytokine Signaling in Immune System” and “Signaling by the B Cell receptor”, while sharing enrichment of “Neutrophil degranulation” with *H. polygyrus* (Supplementary Figure 3B).

### 3 DISCUSSION

Of all known species, only a dozen nematode helminths frequently parasitise humans, yet they infect approximately 2 billion people, nearly a third of the global population. This high prevalence highlights their ability to evade host defences and underscores their potential as immunomodulatory agents of the human immune system [24]. Evidence suggests that the release of ESPs, including soluble proteins, lipids, carbohydrates, and extracellular vesicles, during nematode helminth infections plays a key role in suppressing or regulating inflammatory responses and eosinophilia. This helps create a regulatory or suppressive immune environment that supports

the parasite's long-term survival [25,26]. Despite efforts to identify bioactive molecules, the targets of these molecules and the immunological pathways that helminths exploit to regulate immune responses remain largely unknown. Therefore, elucidating how these ESPs modulate host immune responses may enhance our understanding of host-parasite interactions and support the development of helminth-derived therapeutics for autoimmune and inflammatory diseases.

Here, we used murine macrophages from the RAW 264.7 cell line as a model to investigate the immune response to ESPs from two popular rodent hookworm-like nematodes following stimulation with LPS. Given the intrinsic limitations of RAW 264.7 cells, including their M1-skewed nature, our analysis focused on comparing the relative transcriptional responses elicited by *NbESP* and *HpESP* under identical experimental conditions, rather than on defining absolute polarization states. TNF- $\alpha$  levels significantly decreased following treatment with dexamethasone and *HpESP*, as validated by two techniques, while reduction of TNF-levels by *NbESP*s was not significant. ESPs released by *N. brasiliensis* L3 larvae have been shown to modulate TNF- $\alpha$  levels rat models of LPS-induced pulmonary inflammation [27,28]. Similarly, *N. brasiliensis* adult ESPs can significantly reduce TNF- $\alpha$  levels in bone marrow-derived macrophages (BMDM) from LPS-treated C57BL/6 mice [29]. While using isolated cells like the RAW 264.7 cell line has inherent limitations compared to studying immune responses in whole organisms, this model is widely used to investigate the immunomodulatory potential of helminth ESPs due to its key role in immune activation. Furthermore, it is well known that *N. brasiliensis* secretes different proteins throughout its life cycle, including during the L3 and adult stages [15], which can influence their immunomodulatory effects and, consequently, the *in vitro* responses. A decrease in other pro-inflammatory cytokines (IL-6 and MCP-1) was also observed after treatment with *HpESP* and *NbESP*s. Experiments performed in BMDMs showed that EVs present in the *HpESP* could suppress the secretion of IL-6 and TNF- $\alpha$  after LPS stimulation [30]. In this sense, previous studies showed that IL-6<sup>-/-</sup> mice infected with nematodes had a higher parasite burden, indicating that a decrease in IL-6 expression at the macrophage level is considered beneficial for the parasite [31].

Production of anti-inflammatory and regulatory cytokines was also evaluated. IL-4 (Figure 2A) showed a significant increase in the LPS + Dx group, while ESP-treated groups exhibited only a slight, non-significant trend. In contrast, TGF- $\beta$  (Figure 2B) levels were significantly elevated in both the LPS + Dx and *NbESP* + LPS groups. The differential cytokine responses observed

between the *NbESP* + LPS and *HpESP* + LPS groups may reflect differences in the molecular composition of their respective ESPs. Regarding the results for IL-10 (Figure 1E), no significant differences were observed in any of the cases. This finding is consistent with previous studies that noted that the extracellular vesicles of *N. brasiliensis* (*Nb-EVs*) promoted higher levels of IL-10 secretion compared to ESPs depleted of EVs [32]. Alternatively, it is possible that the release of this cytokine occurs at a later time point, and thus its measurement at 6 hours post-stimulation may not accurately capture its entire expression dynamics. Further studies are warranted to clarify this temporal pattern.

In addition to pro- and anti-inflammatory cytokines, Th1/Th2 markers were also analyzed to further characterize the type of macrophage response. Treatment of RAW264.7 macrophages with *H. polygyrus* ESPs induced a polarization mainly towards Th2, while treatment with *N. brasiliensis* ESPs presented a Th1/Th2 balance. In the case of genes involved in M2 polarization, a higher expression of *Ym1* and *ARG1* levels was observed, correlating with a decrease in pro-inflammatory cytokine levels, such as IL-6, MCP-1, TNF- $\alpha$ , and iNOS. These results suggest that *H. polygyrus* ESPs promote alternative activation of macrophages by shifting the M1/M2 ratio. This coincides with studies by other authors who observed a Th2 polarization induced by this intestinal parasite [33]. In the case of *N. brasiliensis*, an increase in iNOS gene expression was observed, an enzyme involved in macrophage polarization towards the pro-inflammatory M1 profile, while no significant changes were found in TNF- $\alpha$  levels quantified by ELISA or in IL-12p70 gene expression. This may also be associated with a polarization towards inflammatory responses. However, it is important to note that an increase in *Ym1* gene expression, typically expressed in M2 cells, was observed, and a decrease in IL-6 was noted. Previous studies have demonstrated alternative activation of macrophages in the lungs of mice following infection with *N. brasiliensis*, accompanied by increased expression of YM-1, ARG, and other Th2-associated markers. However, it has been speculated that such responses may only be sustained in the presence of functional T cells [34], which could partially explain why we did not observe a purely Th2-skewed response. These findings support the notion that *N. brasiliensis*, similar to other intestinal nematodes such as *Trichinella spiralis*, elicits a mixed Th1/Th2 immune response in macrophages [35,36].

Notably, when administered alone, in the absence of LPS co-stimulation, *NbESP* retained the capacity to induce a broad polarisation programme spanning both M1- (iNOS) and M2-associated

(Ym1, ARG) markers, whereas *Hp*ESP showed little independent polarising activity. This indicates that the pronounced anti-inflammatory effect of *Hp*ESP observed throughout this study does not simply reflect an intrinsic, LPS-independent drive towards alternative activation, but rather an active suppression of an already established pro-inflammatory response. This observation supports the notion that *Nb*ESP and *Hp*ESP achieve broadly convergent anti-inflammatory outcomes under LPS stimulation through distinct underlying mechanisms.

These slight differences in M1/M2 polarization and cytokine production of RAW 264.7 cells after treatment with *N. brasiliensis* and *H. polygyrus* ESPs, were also reflected in the proteomic analysis. The PCA plot revealed distinct clustering of the three groups, with minimal overlap between *N. brasiliensis*-treated, *H. polygyrus*-treated, and control samples. This clear separation suggests that each helminth species induces a unique proteomic signature in macrophages, reflecting species-specific modulation of host immune responses. These findings support the hypothesis that helminth-derived ESPs differentially influence macrophage function, potentially through distinct molecular pathways, in agreement with the cytokine profiles and M1/M2 marker expression also observed in this study. Our study revealed a marked innate immune response by murine macrophages against both *N. brasiliensis* and *H. polygyrus*, as indicated by the central cluster in Figure 4A, which is consistent with previous studies on immune response against helminths [37,38].

Interestingly, while both nematodes exhibited a significant anti-inflammatory effect on murine macrophages, *N. brasiliensis* appeared to act more specifically on proteins involved in anti-inflammatory responses, whereas *H. polygyrus* affected only three of these proteins directly. This suggests that *H. polygyrus* and *N. brasiliensis* may employ different pathways to exert its immunomodulatory effects. For instance, it has been previously shown that *H. polygyrus* secretes a TGF- $\beta$  mimic (*Hp*-TGM) that activate mouse and human Foxp3<sup>+</sup> Treg cells and can expand the host Treg population [20]. Furthermore, two additional TGF- $\beta$  mimics from *H. polygyrus* have been shown to be functionally active [39]. However, to the best of our knowledge, no similar molecules have yet been identified in *N. brasiliensis*. Based on these findings, it is tempting to speculate that *H. polygyrus* may exert a more direct influence on cytokine release, particularly TGF- $\beta$ , whereas *N. brasiliensis* appears to act more indirectly, affecting proteins involved in anti-inflammatory responses, innate immune signalling, neutrophil degranulation, and Toll-like receptor pathways.

Furthermore, it is worth noting that *N. brasiliensis* ESPs have an effect on proteins involved in the “purine metabolism” pathway (e.g., CTP synthase 1, Inosine-5'-monophosphate dehydrogenase, AMP deaminase 3, Adenosine deaminase and Purine nucleoside phosphorylase). While this might indicate the activation of macrophage proliferation, some of these proteins have been shown to act as a positive regulator of T-cell coactivation and to play a crucial role in the proliferation of activated lymphocytes in humans [40,41]. Findings from Wikipathways and Reactome analyses also indicate that *H. polygyrus* predominantly modulates pathways related to humoral immunity, complement activation, and innate immune effector functions, whereas *N. brasiliensis* preferentially influences cytokine- and interleukin-mediated signalling pathways, including B-cell receptor signalling. Together, these results highlight distinct immunomodulatory profiles for the two species, supporting different mechanisms underlying their anti-inflammatory effects.

This study presents several limitations, including the use of a cell line that does not recapitulate the full physiological dynamics of infection. Secondly, the use of adult ESPs at a specific concentration and the study of cytokine profile under a specific timing provides only a specific picture of the full effects promoted by these two nematodes. Thus, the results observed should be interpreted as a relative response within this *in vitro* system, rather than as a direct reflection of the polarization dynamics that occur *in vivo* during *N. brasiliensis* and *H. polygyrus* infection. Furthermore, our experimental design relies on an LPS-driven pro-inflammatory challenge and therefore does not recapitulate the Th2/alarmin-rich context (e.g., IL-33, TSLP, IL-4, IL-13) that predominates during natural helminth infection; combining LPS stimulation with these Th2-associated signals represents an important direction for future work. Similarly, although our design included ESP-alone (LPS-free) treatment groups, validation of these findings in primary bone marrow-derived macrophages (BMDMs), together with a more complete characterisation of M2 marker induction (Arg1, Ym1, Fizz1) in the absence of LPS, will be required to fully establish the intrinsic polarising capacity of *NbESP* and *HpESP*.

Despite the limitations, the results obtained suggest that ESPs from *N. brasiliensis* and *H. polygyrus* exert their anti-inflammatory effects in macrophages through distinct mechanisms. While both nematodes stimulate the production of anti-inflammatory and regulatory cytokines, they appear to activate different pathways involved in immune modulation. This important

divergence should be considered when interpreting results in the context of human helminth infections.

## **4. MATERIALS AND METHODS**

### **4.1 Ethical statement**

All animals were maintained in the animal facility at the National Center for Microbiology in conformity with the Directive on the protection of animals used for scientific purposes (Directive 2010/63/UE, Decision 2020/569/UE and RD 1386/2018). All animal procedures were conducted in accordance with relevant institutional and national guidelines and regulations. The experimental protocols involving animal experiments were approved by the Ethical Animal Experimentation Committee of the Instituto de Salud Carlos III and Comunidad de Madrid (PROEX 124/19 and PROEX 119.3/20). All methods are reported in accordance with the ARRIVE guidelines (<https://arriveguidelines.org/>) for the reporting of animal research.

### **4.2 Parasite material and isolation of ESPs**

*Nippostrongylus brasiliensis* was maintained in C57BL/6 mice (*M. musculus*) purchased from Janvier Labs (Le Genest-Saint-Isle, France). A total of sixty (60) mice were infected subcutaneously with 500 infective larvae (L3) per mouse. Six days post-infection, mice were euthanized by gradual-fill CO<sub>2</sub> inhalation, with chamber volume displaced at a rate of 10–20% per minute in accordance with institutional animal welfare guidelines. Death was confirmed by the absence of respiration and heartbeat and the lack of a corneal reflex, followed by cervical dislocation as a secondary physical method to ensure death. The small and large intestines were then harvested. Adult worms were manually extracted from the small intestine, washed three times with PBS and twice with RPMI 1640 at 37 °C, and then incubated in adult culture medium (RPMI 1640 supplemented with glucose [4.5 g/mL], glutamine [2 mM], and antibiotic/antimycotic [2x]) at 37 °C with 5% CO<sub>2</sub>. A total of 8,000-10,000 worms were cultured at a concentration of 500 worms/mL. The media obtained during the first 24 h after parasite culturing was discarded. Subsequently, excretory-secretory products (ESPs) were collected every 48 hours over a 12-day period and subjected to sequential differential centrifugation at 500, 2,000, and 4,000 g for 30 min each to remove eggs and parasite debris. Finally, media was concentrated using a 3 kDa spin concentrator (Merck Millipore, Billerica, MA, USA) and stored at 1.0 mg/ml in PBS at –80°C until used.

*H. polygyrus* was maintained in NMRI mice (*M. musculus*) purchased from Charles River Laboratories (MA, USA). A total of 10 mice was inoculated orally with  $150 \pm 15$  third-stage larvae (L3) suspended in 0.3–0.4 mL of distilled water. The larval concentration was standardized prior to inoculation using a McMaster counting chamber. Infective L3 larvae were obtained as described previously with minor modifications [42]. Briefly, faecal pellets were collected from day 14 onwards, softened, and homogenized in a mortar with distilled water. The homogenate was centrifuged at 300 rpm for 3 minutes three times, retaining the pellet each time. The pellet was finally resuspended in distilled water and filtered through a mesh strainer and gauze. The filtrate was then centrifuged at 600 rpm for 6 minutes, and the pellet was placed onto the central area of an inverted watch glass covered with pre-moistened filter paper. This assembly was set in a 15 cm diameter Petri dish, with the edges of the filter paper kept in contact with distilled water to maintain humidity. The cultures were incubated at room temperature, in darkness and under high humidity conditions, for 8 days. The liquid phase of the coproculture was collected and the larvae were allowed to sediment for approximately 45 minutes and finally stored in distilled water at 4°C until use. *H. polygyrus* adult worms were collected at day 14 days post infection and cultivated at a concentration of 500 worms/mL as described previously [43]. Finally, media was also concentrated using a 3 kDa spin concentrators and stored at 1.0 mg/ml in PBS at –80°C until used.

#### 4.3 Endotoxin removal

Endotoxin removal was performed using a detergent-based phase separation method [44]. Briefly, Triton X-114 was added to the protein samples to a final concentration of 1% (v/v). Samples were incubated at 4°C for 30 min under continuous agitation, followed by incubation at 37°C for 10 min in a water bath. The mixtures were then centrifuged at 13,000 g at 25°C, and the aqueous phase was carefully recovered. This procedure was repeated two additional times to further reduce endotoxin levels. Residual endotoxin levels were quantified using the Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific) following the manufacturer's protocol. Only protein preparations containing less than 5 EU/mg protein were used in subsequent experiments, thereby minimizing potential endotoxin-mediated immunomodulatory effects.

#### 4.4 Cell culture conditions and treatments

Murine macrophage RAW 264.7 cells (ATCC® TIB-71™) purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) were propagated in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The culture medium, pre-warmed to 37°C, was refreshed every 48 hours, with a total volume of 10 mL per T75 flask.

To benchmark the anti-inflammatory potential of *NbESP* and *HpESP* against LPS-induced inflammation and against a pharmacological reference anti-inflammatory drug (dexamethasone), RAW 264.7 cells were cultured in 6-well plates at the same density and divided into the following eight groups: i) Control group: Cells cultured in supplemented DMEM only; ii) LPS-treated group: Cells stimulated with 5 ng/mL of LPS; iii) Dexamethasone control group: Cells treated with 1 µM dexamethasone; iv) Dexamethasone + LPS intervention group: Cells treated with 1 µM dexamethasone and stimulated with 5 ng/mL of LPS; v) *NbESP*-treated group: Cells treated with 10 µg/mL of *NbESP*; vi) *NbESP* + LPS intervention group: Cells treated with 10 µg/mL *NbESP* and stimulated with 5 ng/mL of LPS; vii) *HpESP*-treated group: Cells treated with 10 µg/mL of *HpESP*; and viii) *HpESP* + LPS intervention group: Cells treated with 10 µg/mL *HpESP* and stimulated with 5 ng/mL of LPS. At time 0, groups were stimulated with LPS. One hour later, dexamethasone, *NbESP* or *HpESP* were added to the wells. Supernatants were collected 6 hours post-LPS stimulation, and cells from each well were harvested by adding cold PBS and centrifuging at 1,000 rpm for 5 minutes. Both supernatants and cell pellets were stored at -20°C until further analysis. Each experimental condition was performed in three independent biological replicates, corresponding to three separately treated wells per condition.

#### 4.5 Cytokine quantification by cytometric bead array and ELISA

The cytokines present in the cell culture supernatants were quantified using the BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Briefly, the beads coated with six specific capture antibodies were mixed for each assay. Subsequently, 50 µL of the mixed capture beads, 50 µL of the sample or standard dilution, and 50 µL of the PE detection reagent were added to each test tube. The tubes were, then, incubated for 2 hours at room temperature (RT) in the dark. After incubation, the samples were washed with 1 mL of wash buffer and centrifuged at 200 g for 5 minutes. The resulting microbead pellet was resuspended in 300 µL of buffer following the removal of the

supernatant. Samples were finally analyzed using the BD Accuri C6 Plus flow cytometer (BD Biosciences) and data were processed with FlowJo software (BD Biosciences).

The TNF- $\alpha$  cytokine levels were further confirmed using the Mouse TNF alpha Uncoated ELISA kit (Invitrogen) following the manufacturer's instructions. Briefly, plates were coated with 100  $\mu$ L/well of capture antibody diluted 1:250 in coating buffer overnight at 4°C. The following day, plates were washed x3 in washing buffer and blocked with 200  $\mu$ L of ELISA/ELISPOT Diluent for 1 h at RT. Plates were washed again and incubated with 100  $\mu$ L of standards or samples for 2 h at RT. After washing again x3 with washing buffer, 100  $\mu$ L/well of Detection Antibody diluted 1:250 was added and plates were incubated for 1 h at room temperature. A wash was performed as described above and finally, 100  $\mu$ L/well of diluted Streptavidin-HRP was added to each well and plates were developed after a final wash using 100  $\mu$ L of 3,3',5,5'-tetramethylbenzidine (TMB) for 15 min, followed by 100  $\mu$ L of stop solution. Plates were read at a wavelength of 450 nm on a Heales mod. MB580 (Shenzhen Huisong Technology Development Co., Ltd. Shenzhen, China) microplate reader.

#### 4.6 RNA extraction, cDNA synthesis and qPCR

Total RNA from cells treated with excretory-secretory products (ESPs) was extracted using the Quick-RNA™ Miniprep Kit (Zymo), following the manufacturer's instructions. The extracted RNA was quantified using a NanoDrop One/One spectrophotometer (ThermoFisher) by measuring absorbance at 260 nm. The purity of the RNA was assessed through absorbance ratios at 260/280 and 260/230 nm. Complementary DNA (cDNA) synthesis was performed using the SuperScript III kit (Thermo Fisher Scientific). The reaction was initiated by mixing 3  $\mu$ L of RNA from each sample, 1  $\mu$ L of oligo(dT) (50  $\mu$ M), 1  $\mu$ L of dNTP Mix (10 mM), and distilled water to a final volume of 13  $\mu$ L. This mixture was incubated in a GeneAmp PCR System 2700 (Applied Biosystems) thermocycler for 5 minutes at 65°C and then for 1 minute at 4°C. Subsequently, 4  $\mu$ L of 5X First-Strand Buffer, 1  $\mu$ L of DTT (0.1 M), and 1  $\mu$ L of RNaseOUT (RNase inhibitor, 40 U/ $\mu$ L) were added to reach a final reaction volume of 20  $\mu$ L. The reaction was incubated for 60 minutes at 50°C, followed by 15 minutes at 70°C to inactivate the enzyme. The synthesized cDNA was quantified using the NanoDrop One/One spectrophotometer (Thermo Fisher Scientific) by measuring absorbance at 260 nm, with purity assessed via 260/280 and 260/230 ratios. The cDNA was stored at -20°C until use.

cDNA amplification was performed using quantitative real-time PCR (qPCR) with the SYBR-Green system. The actin gene was used as an endogenous control, and the expression of *IL-4*, *TGF $\beta$* , *iNOS*, *Ym1*, and *ARG* genes was analyzed. Amplifications were conducted in the presence of SYBR-Green PCR Master Mix (Applied Biosystems) using 2.5  $\mu$ g of template cDNA and 1  $\mu$ L of each primer (forward and reverse; Table 1) in a final reaction volume of 20  $\mu$ L. qPCR reactions were performed on a Rotor-Gene Q (Qiagen) using the following conditions: initial denaturation at 95°C for 10 seconds (1 cycle) followed by 40 cycles of denaturation (95°C for 10 sec), annealing (55°C or 65°C for 15 sec) and extension (72°C for 20 sec). Gene expression was normalised to the housekeeping gene as described before [45,46] and relative expression levels were calculated using the  $2^{-\Delta\Delta C_t}$  method using Control (untreated cells) as a reference group [47].

#### 4.7 Cell processing and protein extraction

Three biological replicates of cells treated with *NbESP* or *HpESP* were lysed using 100  $\mu$ L of RIPA buffer (Invitrogen) following the manufacturer's instructions. Protein extracts (20  $\mu$ g in 100  $\mu$ L of RIPA) were reduced by adding 10  $\mu$ L of 100 mM tris(2-carboxyethyl)phosphine (TCEP) and incubated at 37°C with 600 rpm for 45 minutes. Alkylation was performed by adding 11  $\mu$ L of 400 mM chloroacetamide, followed by a 30-minute incubation at RT in the dark with 600 rpm shaking.

Subsequently, 100  $\mu$ L of SeraMag magnetic beads mix (GE Healthcare, Chicago, IL, USA; 50% hydrophilic beads and 50% hydrophobic beads) and 200  $\mu$ L of acetonitrile (ACN) were added to facilitate protein binding to the beads. The mixture was incubated at RT with 600 rpm for 35 minutes. Supernatants were removed, and magnetic beads were washed twice with 70% ethanol and once with ACN. Proteins were then digested overnight at 37°C with 0.5  $\mu$ g of porcine trypsin (Thermo Fisher Scientific) in 100  $\mu$ L of 200 mM HEPES (pH 8.0) with 600 rpm. The following day, samples were sonicated twice, and the resulting supernatants were dried under vacuum in preparation for desalting.

The Pierce C18 Spin Tips (Thermo Fisher Scientific) were used for peptide purification. The dried peptides were resuspended in 50  $\mu$ L of 0.1% trifluoroacetic acid (TFA) and loaded onto the C18 Spin Tip, followed by centrifugation at 1,000–2,000 g to allow peptides to bind to the hydrophobic matrix. To remove contaminants, 20  $\mu$ L of 0.1% TFA was added for washing. Peptides were eluted using 20  $\mu$ L of 0.1% TFA in 80% ACN, with centrifugation at 1,000 g for 1

minute. The eluted peptides were dried under vacuum and stored at  $-80^{\circ}\text{C}$  until analysis by LC-MS/MS.

#### 4.8 Mass spectrometry and protein identification

For LC-MS/MS, peptides were analyzed in an Orbitrap Astral mass spectrometer coupled to a Vanquish Neo UHPLC System (Thermo Fisher Scientific). Peptide samples were loaded into the precolumn PepMap Trap Catridge  $5\text{ }\mu\text{m}$ ,  $300\text{ }\mu\text{m} \times 5\text{ mm}$  (Thermo Fisher Scientific) and eluted in an Easy-Spray PepMap RSLC C18  $2\text{ }\mu\text{m}$ ,  $50\text{ }\mu\text{m} \times 15\text{ cm}$  (Thermo Fisher Scientific) heated at  $50^{\circ}\text{C}$ . The mobile phase flow rate was  $300\text{ nL/min}$  and  $0.1\%$  formic acid (FA) in  $\text{H}_2\text{O}_{\text{mq}}$  and  $0.1\%$  FA in  $80\%$  acetonitrile (ACN) were used as elution buffers A and B, respectively. The  $15\text{ min}$  elution gradient was:  $4\%-10\%$  buffer B for  $2\text{ min}$ ,  $10\%-40\%$  buffer B for  $11\text{ min}$ ,  $40\%-99\%$  buffer B for  $0.5\text{ min}$ , and  $99\%$  buffer B for  $1.5\text{ min}$ . Prior to injection, samples were re-suspended in  $10\text{ }\mu\text{L}$  of buffer A, and  $1\text{ }\mu\text{L}$  of each sample were injected, and analyzed in data independent acquisition (DIA) mode. For ionization,  $1900\text{ V}$  of liquid junction voltage and  $280^{\circ}\text{C}$  capillary temperature was used. The full scan method employed a  $m/z\text{ }380\text{-}980$  mass selection, an Orbitrap resolution of  $240000$  (at  $m/z\text{ }200$ ), an automatic gain control (AGC) value of  $500\%$ , and maximum injection time (IT)  $5\text{ ms}$ . The MS/MS was performed with the Astral mass analyzer, using an AGC of  $500\%$ , an IT of  $3\text{ ms}$ , and a normalized collision energy (NCE) of  $25$  for fragmentation of precursors. The scan range was set from  $380$  to  $980\text{ m/z}$ , with an isolation window of  $2\text{ m/z}$ , and window placement optimization was enabled. Thus, a total of  $299$  windows were analyzed in each cycle.

A directDIA search was performed using Spectronaut v19.4 against a concatenated target/decoy database consisting of the mouse proteome ( $17,216$  reviewed proteins from UP000000589 accessed in March 2024) and common contaminants ( $246$  proteins from cRAP database accessed in October 2024) as well as proteomes from *N. brasiliensis* (PRJNA994163;  $29,473$  proteins) and *H. polygyrus* (PRJEB15396;  $25,212$  proteins) downloaded from WormBaseParasite in November 2024 (WBPS19). Enzyme was set to Trypsin/P, fixed modifications to Carbamidomethyl (C) and variable modifications to Acetyl (Protein N-term) and Oxidation (M). Precursor qvalue and PEP cutoffs were set to  $0,01$  and  $0,2$ , respectively, while protein qvalue cutoff was set to  $0,01$ . Peptide and protein FDR were set to  $0,01$ . Single hit proteins were excluded from the analysis. Mass spectrometry data along with the identification results and search parameters have been deposited

in the ProteomeXchange Consortium *via* the PRIDE partner repository [48] with the dataset identifier PXD062974.

#### 4.9 Gene Ontology and PPI analyses

The GO information for each protein was retrieved from Uniprot database ([www.uniprot.org](http://www.uniprot.org)). Furthermore, an enrichment analysis was performed using STRINGdb using the default settings except for the minimum required interaction score, which was set to “high confidence” (0.7). The interactome graph involving “immune system response” proteins was depicted using the python NetworkX library (<https://networkx.org/>). Reactome and Wikipathways terms were grouped by similarity  $\geq 0.8$  and sorted by signal.

#### 4.10 Statistical and bioinformatic analyses

For the CBA, ELISA and qPCR analyses, statistical significance was calculated using Student's t-test by comparing the LPS-treated group to each treatment or control. \*\*\*\* =  $p < 0.0001$ ; \*\*\* =  $p < 0.001$ ; \*\* =  $p < 0.01$ ; \* =  $p < 0.05$ ; no value =  $p > 0.05$ . For the proteomics data, a DIA quantitative analysis was performed following protein identification using Spectronaut® v19.4. Quantitative results were normalised by cross-run normalization of medians. An unpaired t-test was performed to compare protein abundance between groups after log2 transformation. To account for multiple hypothesis testing, raw p-values were corrected using the Benjamini-Hochberg (BH) procedure, and a false discovery rate (FDR) threshold of 5% (Qvalue  $< 0.05$ ) was applied to identify significantly regulated proteins.. Fold-changes were considered as significant when  $\geq 0.58$  and adjusted  $p$ -value (Qvalue)  $\leq 0.05$ . Volcano and Principal Component Analysis (PCA) plots were performed using the ggplot2 R package using customised scripts. All scripts are available and can be downloaded from <https://github.com/JavierSotillo/Scripts>.

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#### Data availability

Mass spectrometry data along with the identification results and search parameters have been deposited in the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD062974. Reviewer can access the dataset by logging in to the PRIDE website using the following account details:

**Username:** reviewer\_pxd062974@ebi.ac.uk

**Password:** Tm9dBo09W50t

### Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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### Figure legends

**Figure 1.** Cytokine levels in the supernatants or cell lysates of RAW264.7 murine macrophages. RAW264.7 cells were stimulated with LPS and treated with excretory/secretory products from *Nippostrongylus brasiliensis* (LPS + NbESP), *Heligmosomoides polygyrus* (LPS + HpESP) or with dexamethasone as positive control (LPS + Dx). Levels of TNF- $\alpha$  (A), MCP-1 (B), IL-6 (C), IL-12p (D), TGF- $\beta$  (E), IL-10 (F) and IL-4 (G) were quantified using the CBA Mouse Inflammation Kit and/or by qPCR as indicated. Data are shown as mean  $\pm$  SD of three independent biological replicates (three separate wells per condition), each analysed in triplicate (technical replicates). Individual data points represent biological replicates.

**Figure 2.** Gene expression measured by qPCR in RAW264.7 murine macrophages following stimulation with LPS and treatment with excretory/secretory products from *Nippostrongylus brasiliensis* (LPS + NbESP) or *Heligmosomoides polygyrus* (LPS + HpESP) or with dexamethasone as positive control (LPS + Dx). Gene expression of YM-1 (A), Arginase (B) and iNOS (C) were measure by qPCR and analysed following the  $2^{-\Delta\Delta C_t}$  method.

**Figure 3.** Proteomics analysis of RAW264.7 cells after incubation with *Nippostrongylus brasiliensis* (NbESP) or *Heligmosomoides polygyrus* (HpESP) excretory/secretory products. (A) Principal component analysis of each of the three replicates of RAW264.7 cells following treatment with NbESP and HpESP. Volcano plot representing all proteins identified in RAW264.7 cells after incubation with HpESP (B) and NbESP (C). Purple and cyan nodes represent upregulated and downregulated proteins, respectively.

**Figure 4.** Analysis of immune-related proteins differentially regulated by *N. brasiliensis* and *H. polygyrus*. (A) Protein-protein interaction network showing clusters grouped according to biological processes. WikiPathways enrichment analysis of proteins annotated with the GO term “Immune system” and differentially regulated by *H. polygyrus* (B) and *N.brasiliensis* (C).

### Supplementary material legends

**Supplementary Figure 1.** TNF- $\alpha$  levels in the supernatant of RAW264.7 murine macrophages measured by ELISA. RAW264.7 cells were stimulated with LPS and treated with excretory/secretory products from *Nippostrongylus brasiliensis* (LPS + NbESP) or *Heligmosomoides polygyrus* (LPS + HpESP) or with dexamethasone as positive control (LPS + Dx).

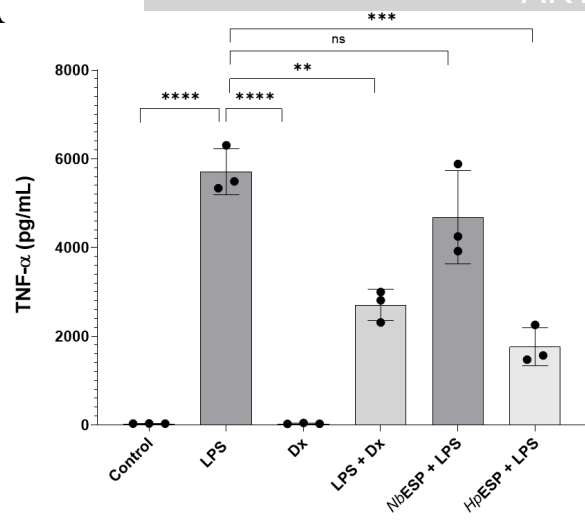
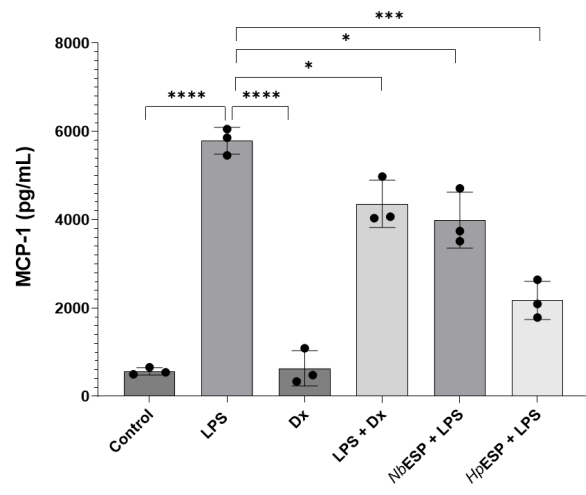
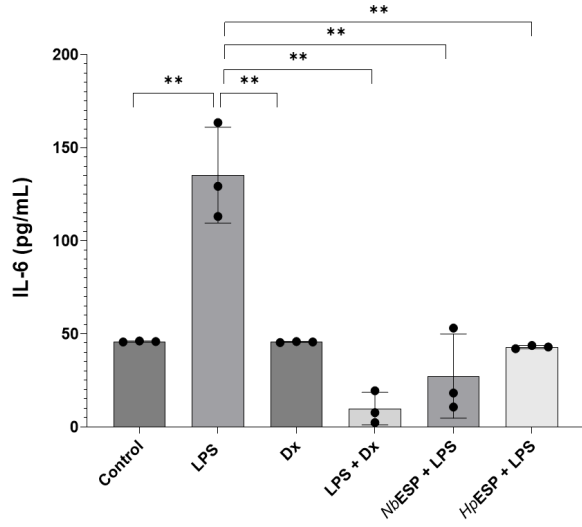
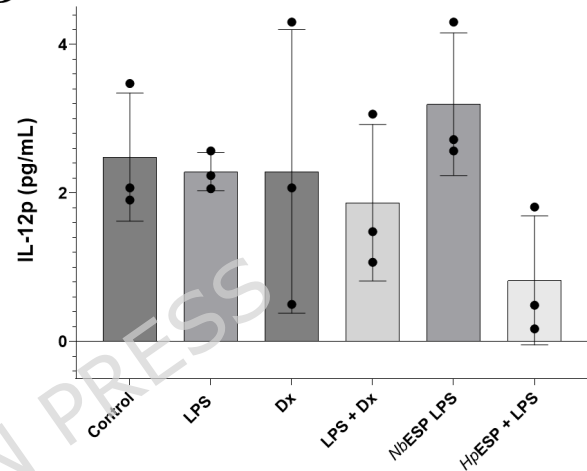
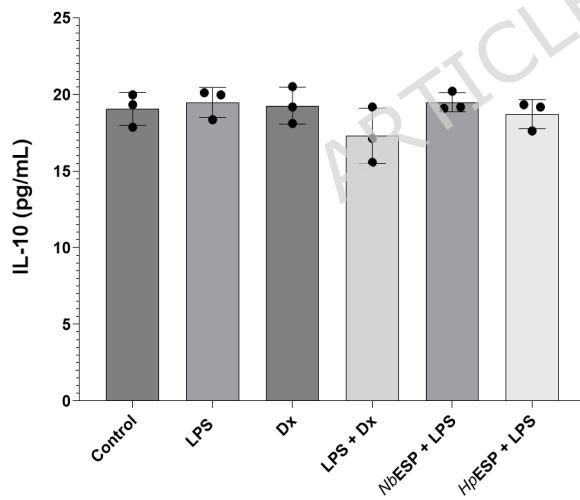
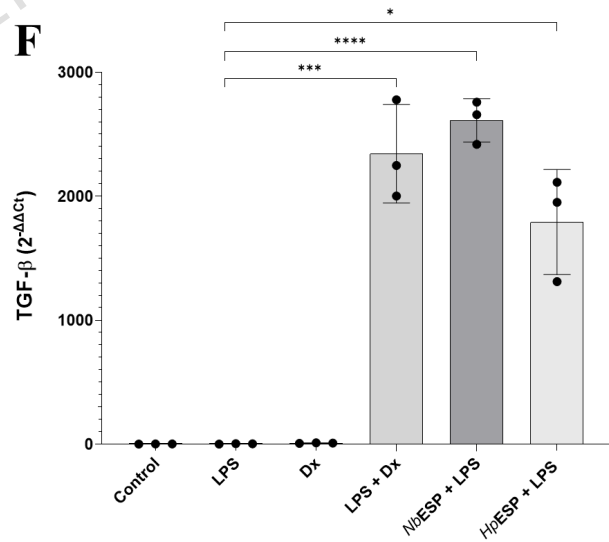
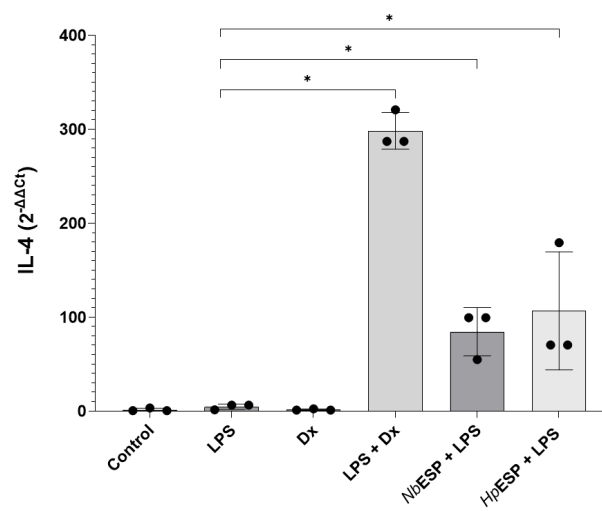
**Supplementary Figure 2.** Gene expression measured by qPCR in RAW264.7 murine macrophages following treatment with excretory/secretory products from *Nippostrongylus brasiliensis* (NbESP) or *Heligmosomoides polygyrus* (HpESP). Gene expression of YM-1 (A), Arginase (B) and iNOS (C) were measure by qPCR and analysed following the  $2^{-\Delta\Delta C_t}$  method.

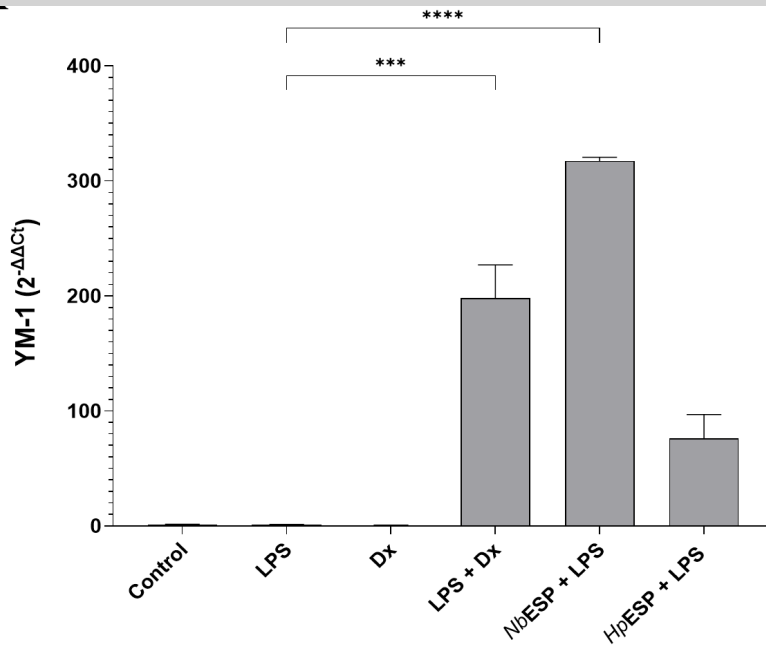
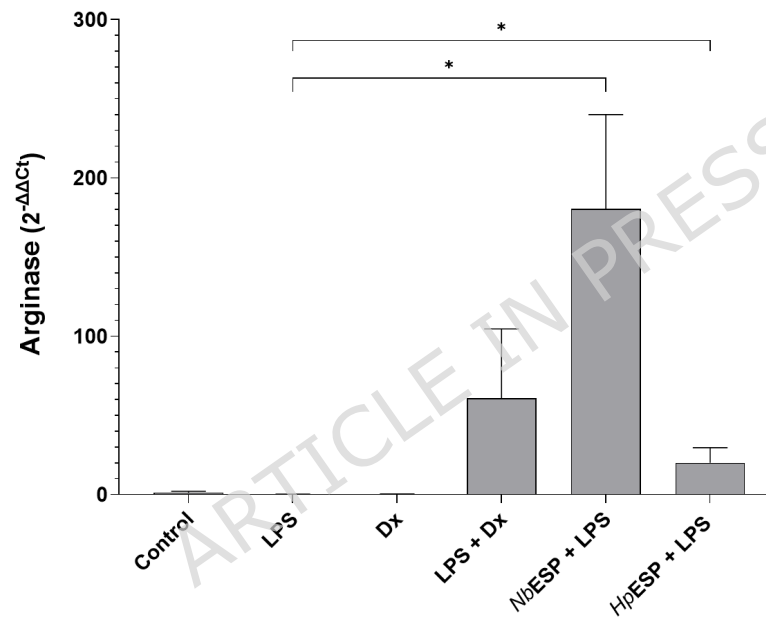
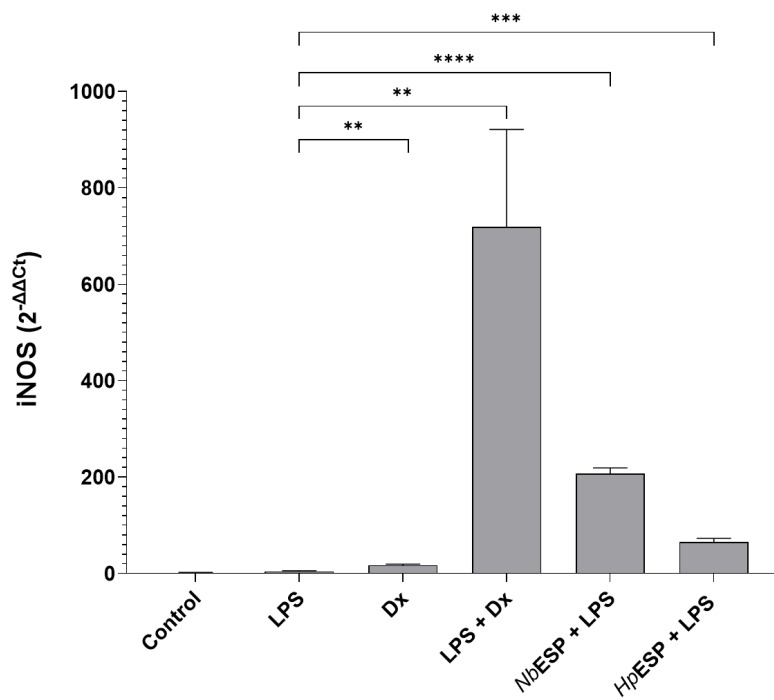
**Supplementary Figure 3.** Reactome enrichment analysis of proteins annotated with the GO term “Immune system” and differentially regulated by *H. polygyrus* (A) and *N.brasiliensis* (B).

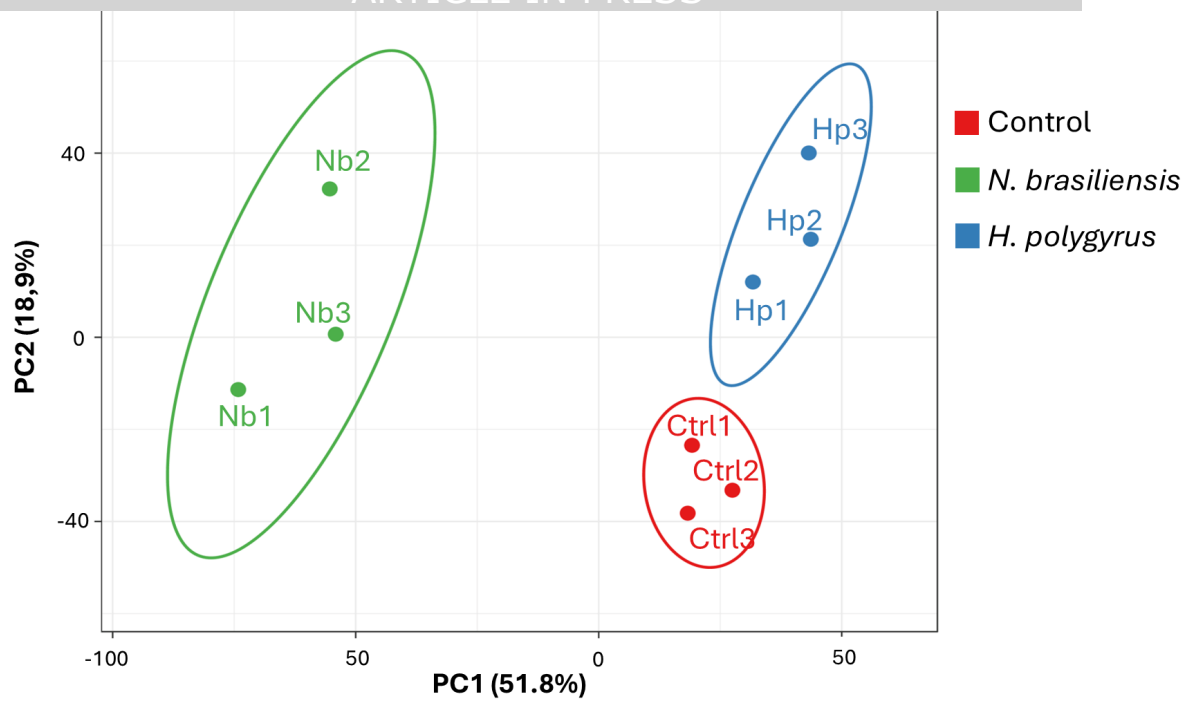
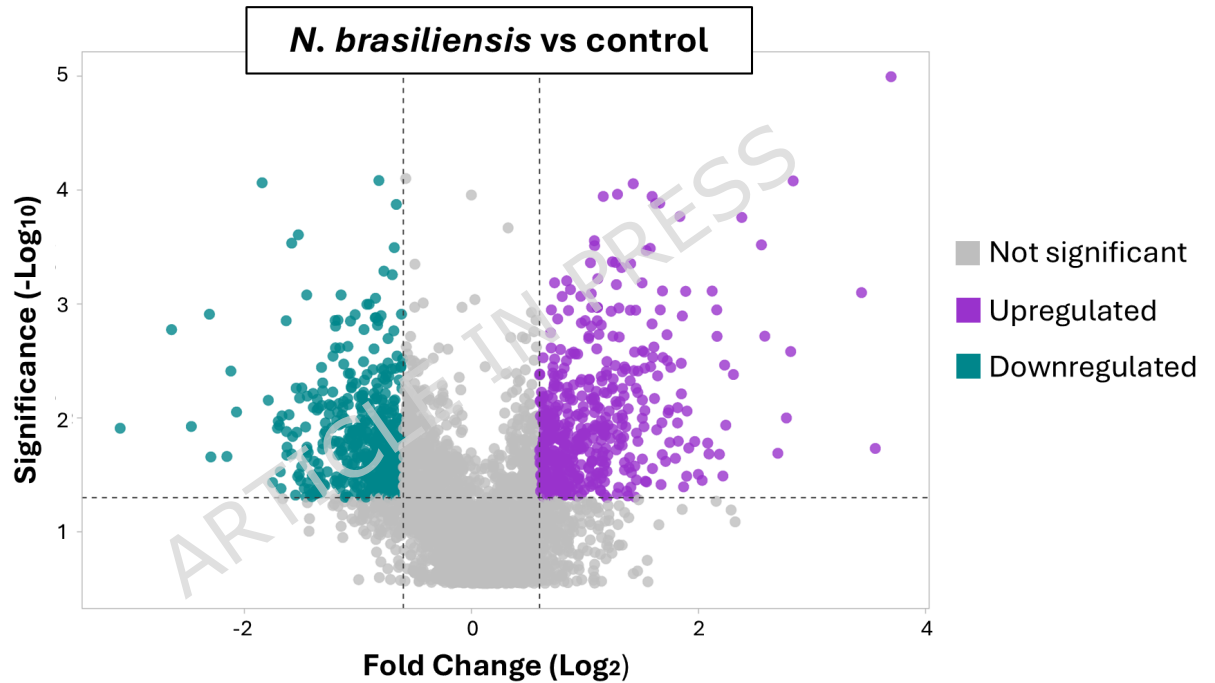
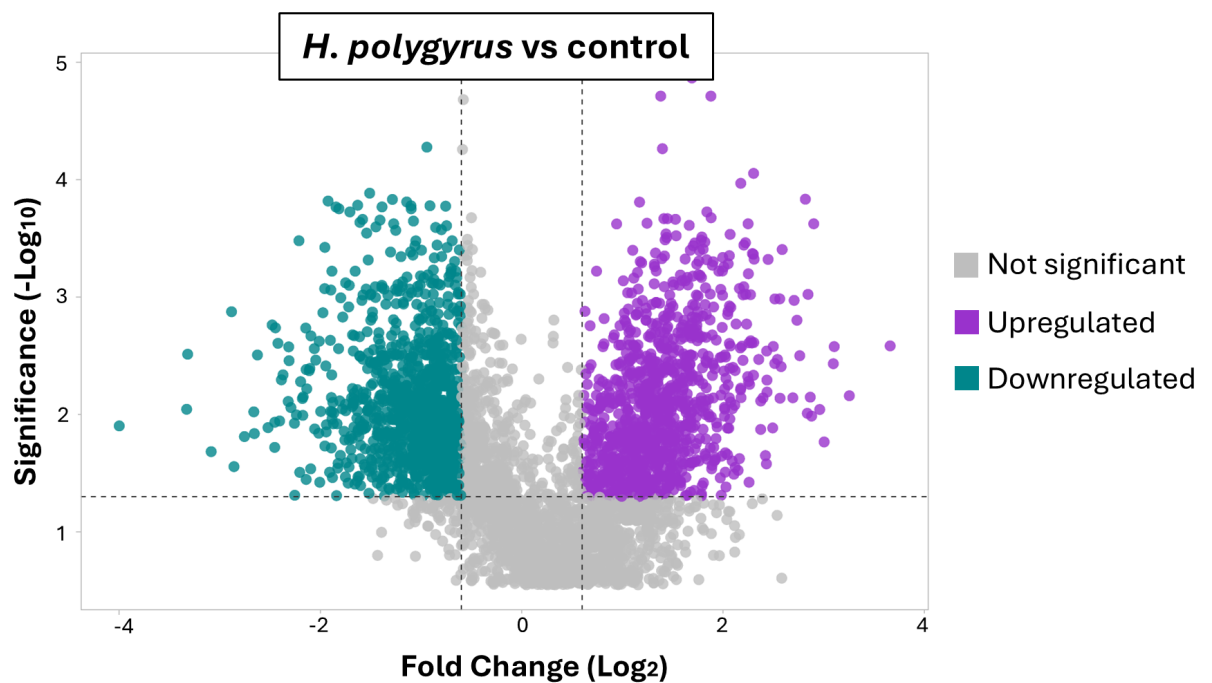
### Tables

**Table 1:** Oligonucleotides Used for Gene Expression Analysis via SYBR-Green qPCR

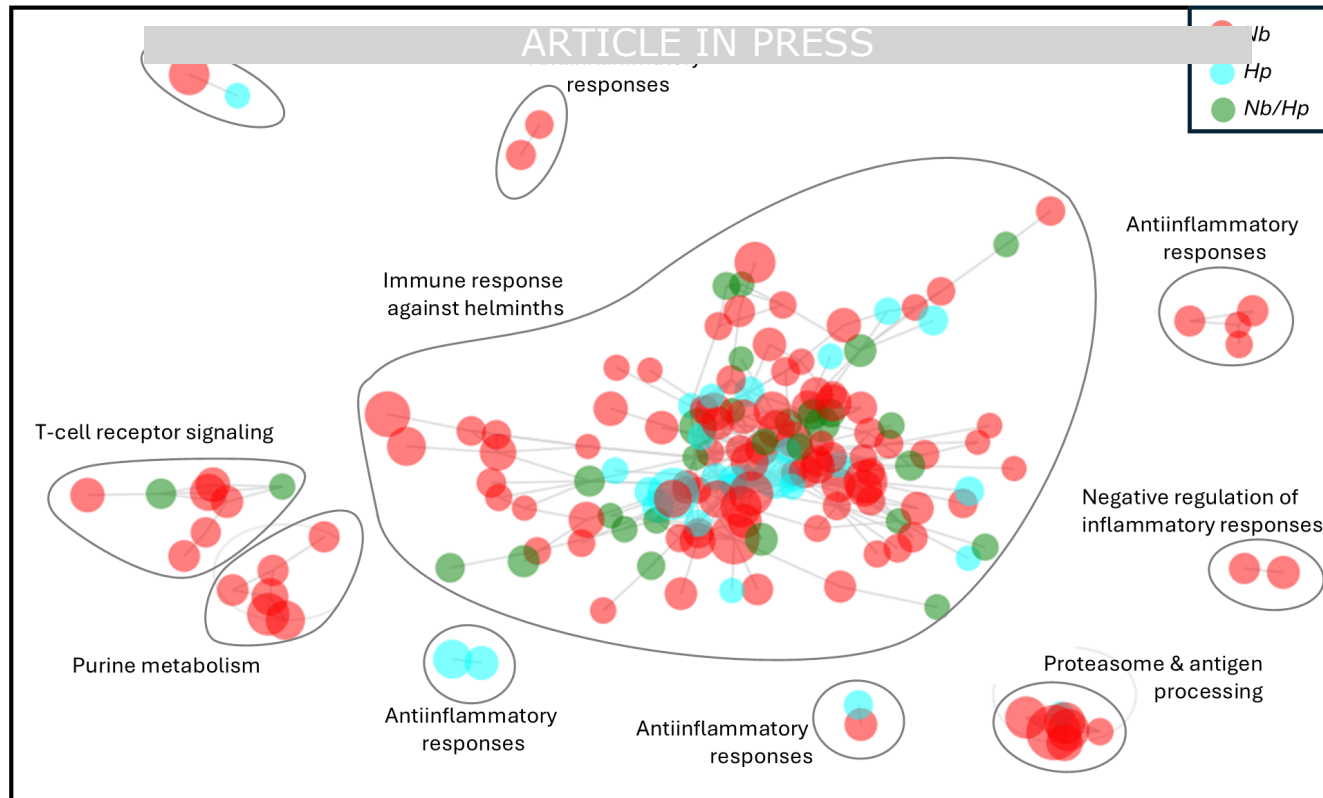
| GENE        | PRIMER FORWARD                 | PRIMER REVERSE                 |
|-------------|--------------------------------|--------------------------------|
| ACT         | 5'-CGGTTCCGATGCCCTGAGGCTCTT-3' | 5'-CGTCACACTTCATGATGGAATTGA-3' |
| IL-4        | 5'-CCCCAGCTAGTTGTCATCC-3'      | 5'-AGGACGTTTGGCACATCCAT-3'     |
| iNOS        | 5'-CAGCTGGGCTGTACAAACCTT-3'    | 5'-CATTGGAAGTGAAGCGTTTCG-3'    |
| TGF $\beta$ | 5'-TTGCTTCAGCTCCACAGAGA-3'     | 5'-TGGTTGTAGAGGGCAAGGAC-3'     |
| Ym1         | 5'-TTTCTCCAGTGTAGCCATCCTT-3'   | 5'-TCTGGGTACAAGATCCCTGAA-3'    |
| ARG         | 5'-TTTTTCCAGCAGACCAGCTT-3'     | 5'-AGAGATTATCGGAGCGCCTT-3'     |

**A****B****C****D****E****F****G**

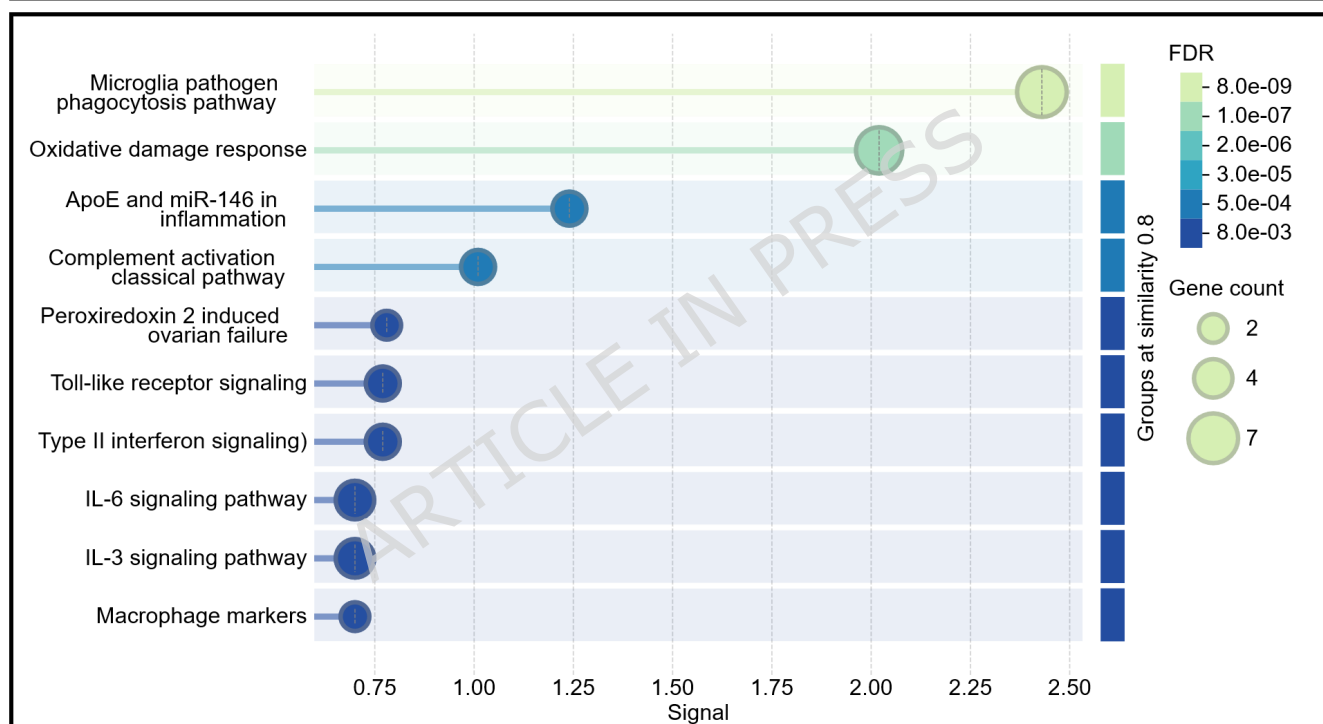
**B**

**A****B****C**

A



B



C

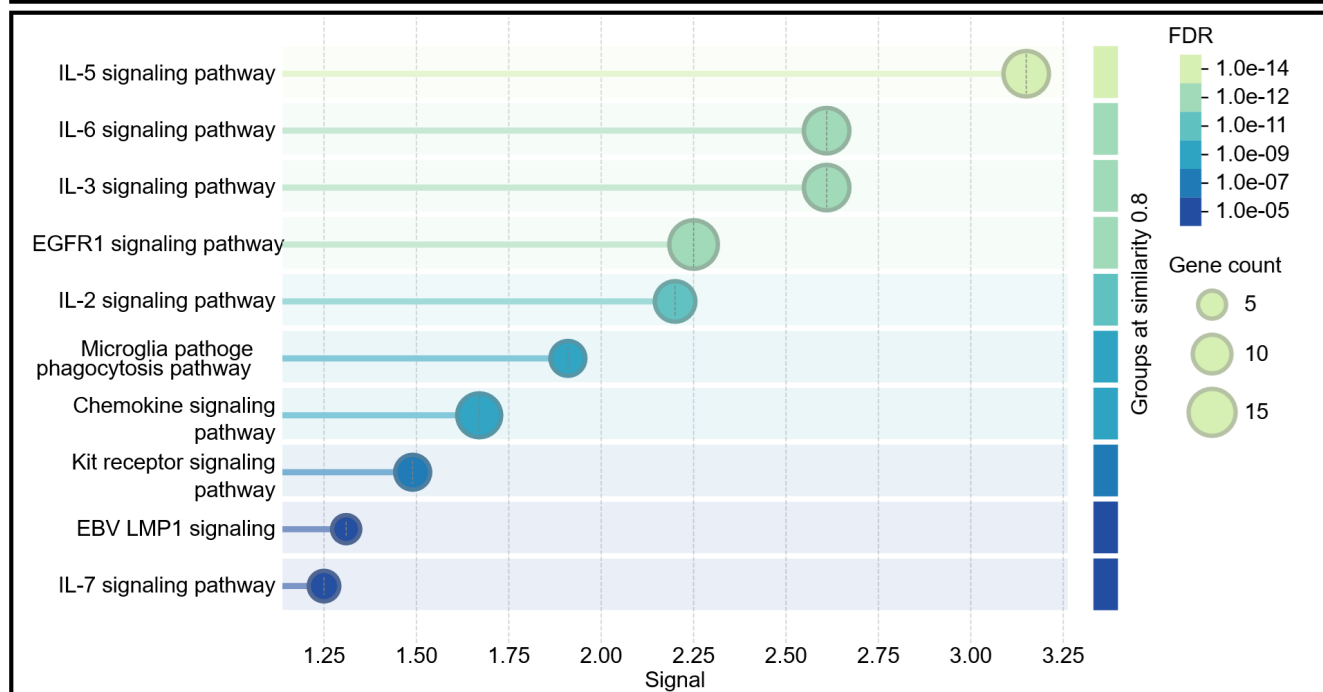


**Table 1:** Oligonucleotides Used for Gene Expression Analysis via SYBR-Green qPCR

| <b>GENE</b>                   | <b><i>PRIMER FORWARD</i></b>   | <b><i>PRIMER REVERSE</i></b>   |
|-------------------------------|--------------------------------|--------------------------------|
| <b>ACT</b>                    | 5'-CGGTTCCGATGCCCTGAGGCTCTT-3' | 5'-CGTCACACTTCATGATGGAATTGA-3' |
| <b>IL-4</b>                   | 5'-CCCCCAGCTAGTTGTCATCC-3'     | 5'-AGGACGTTTGGCACATCCAT-3'     |
| <b>iNOS</b>                   | 5'-CAGCTGGGCTGTACAAACCTT-3'    | 5'-CATTGGAAGTGAAGCGTTTCG-3'    |
| <b>TGF <math>\beta</math></b> | 5'-TTGCTTCAGCTCCACAGAGA-3'     | 5'-TGGTTGTAGAGGGCAAGGAC-3'     |
| <b>Ym1</b>                    | 5'-TTTCTCCAGTGTAGCCATCCTT-3'   | 5'-TCTGGGTACAAGATCCCTGAA-3'    |
| <b>ARG</b>                    | 5'-TTTTTCCAGCAGACCAGCTT-3'     | 5'-AGAGATTATCGGAGCGCCTT-3'     |