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Biphasic role of L-TGF-β2 in modulating lamprey inflammation: Insights into vertebrate immune evolution

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ABSTRACT

The regulation of inflammatory balance is central to immune homeostasis, with disruptions contributing to autoimmune diseases. As representatives of early vertebrates, lampreys offer a valuable model for investigating the evolutionary foundations of immune regulation. This study examines the dual role of L-TGF-β2 in modulating inflammation in lampreys, providing insights into the evolutionary origins of immune homeostasis mechanisms. Using lipopolysaccharide (LPS)-induced inflammation models, recombinant L-TGF-β2 was found to enhance leukocyte chemotaxis in quiescent states while inhibiting excessive migration during activation. Quantitative PCR revealed that L-TGF-β2 stimulates pro-inflammatory cytokine expression during early inflammatory phases and attenuates it during resolution. Tissue-specific expression patterns of TGF-β receptors indicated their potential role in mediating the distinct regulatory effects of L-TGF-β2 across diverse immune environments. These findings align with the biphasic functions of TGF-β observed in higher vertebrates, underscoring the evolutionary conservation of this signaling pathway. This research enhances understanding the functional versatility of TGF-β signaling and its pivotal role in vertebrate immune evolution, providing insights into ancient mechanisms of immune homeostasis and their modern implications for inflammatory disease research.

1. Introduction

During inflammation, immune cells release and receive pro-inflammatory or anti-inflammatory signals, and the dynamic balance between these signals determines the progression of the inflammatory response. When this balance is disrupted, chronic inflammation can occur, potentially leading to autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. Investigating the differences and evolutionary aspects of immune homeostasis regulation across species can elucidate the regulatory networks of the immune system and the pathological mechanisms underlying immune dysregulation (Ning et al., 2019).

In higher vertebrates, transforming growth factor-β (TGF-β) is a critical immune-regulatory molecule that demonstrates biphasic effects contingent on cell type and differentiation status (Letterio and Roberts, 1998). During the early stages of inflammation, TGF-β establishes concentration gradients that facilitate immune cell migration to inflammatory sites, while concurrently upregulating pro-inflammatory cytokine expression and activating immune cells. Conversely, TGF-β contributes significantly to inflammation resolution by inhibiting chemokine and pro-inflammatory cytokine expression in macrophages. An imbalance between the pro-inflammatory and anti-inflammatory activities of TGF-βs can lead to chronic inflammation and tissue fibrosis, as demonstrated in animal models.

TGF-β homologs are unique to deuterostomes. Lower deuterostomes, such as tunicates (*Urochordata*) and amphioxus (*Cephalochordata*), possess a single TGF-β homolog, whereas higher vertebrates have evolved three distinct homologs: TGF-β1, TGF-β2, and TGF-β3. Although the role of TGF-β in maintaining immune homeostasis in higher vertebrates is well-documented, the evolutionary timeline of their acquisition of immune-regulatory functions remains elusive (Herpin et al., 2004). Lampreys, as extant jawless vertebrates, possess an immune system analogous to that of jawed vertebrates, rendering them a valuable model for studying immune system evolution. Lampreys possess two genes, L-TGF-β2 and L-TGF-β3, homologous to TGF-β2 and TGF-β3 in higher vertebrates, as evidenced by prior studies (Liu et al., 2022). Notably,

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L-TGF-β2 expression is markedly upregulated following lipopolysaccharide (LPS) stimulation, indicating its pivotal role in innate immune regulation in lampreys. Recombinant L-TGF-β2 protein exhibits context-dependent regulatory effects on immune cell proliferation and apoptosis. Nevertheless, it remains uncertain whether TGF-β in lampreys exhibits the biphasic functions of promoting and inhibiting inflammation observed in higher vertebrates.

In this study, we employed a lamprey inflammation model to examine the effects of L-TGF-β2 on leukocyte chemotaxis and pro-inflammatory cytokine expression, while also investigating TGF-β receptor expression patterns across various immune tissues. These findings underscore the critical role of TGF-β2 in the innate immune response of lampreys and offer novel insights into the evolution of immune homeostasis regulatory mechanisms.

2. Materials and methods

2.1. Animal maintenance and cell culture

Healthy adult Japanese lampreys (*Lampetra japonica*) of both sexes, ranging from 30 to 50 cm in length, were maintained in fiber-reinforced plastic (FRP) tanks with a recirculating water system at 10 °C. For immune stimulation, each lamprey received an intraperitoneal injection of 100 μg lipopolysaccharide (LPS; Sigma-Aldrich) or an equivalent volume of physiological saline as a control. Twelve hours post-injection, the animals were anesthetized with MS222 (100 mg/L; Aladdin) prior to sample collection. All animal handling and experimental procedures were conducted in compliance with the ethical guidelines approved by the Animal Welfare and Research Ethics Committee of the Institute of Dalian Medical University (Permit Number: SYXK2008-0002).

Peripheral blood leukocytes were isolated following a previously described protocol (Liu et al., 2022). Briefly, blood was collected from the cardiac region of lampreys and subjected to Ficoll-Histopaque gradient centrifugation at 400×*g* for 30 min to isolate the leukocyte fraction. Following cell counting with a hemocytometer and trypan blue exclusion to assess viability, leukocytes were cultured in Leibovitz L-15 medium (Meilunbio) supplemented with 10 % fetal bovine serum (HyClone), antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin), and 2 mM L-glutamine (Meilunbio). Leukocytes were cultured at a density of 1–2 × 10^6 cells/mL in a low-temperature incubator at 18 °C for subsequent experiments.

2.2. Boyden chamber assay

Recombinant L-TGF-β2 (rL-TGF-β2) was expressed in an *Escherichia coli* system and purified as previously described (Liu et al., 2022). Briefly, the mature peptide sequence of L-TGF-β2 was cloned into the pET28a expression vector. The recombinant protein was then induced, purified, and refolded to produce the final product. Endotoxin levels were evaluated using an endotoxin detection kit (Xiamen Bioendo Technology), confirming concentrations below 1 EU/μg. To assess the effect of rL-TGF-β2 on quiescent lamprey leukocyte migration, a Boyden chamber assay was performed using a 24-well Transwell apparatus with 5.0 μm pore polycarbonate membrane inserts (Cat. 3421; Corning). Briefly, freshly isolated leukocytes from Japanese lampreys were resuspended in low-serum medium (Leibovitz L-15 medium supplemented with 0.1 % fetal bovine serum, antibiotics, and 2 mM L-glutamine) at a density of 1 × 10^6 cells/mL. The lower Transwell compartment was filled with 500 μL of medium containing either PBS (negative control), 500 ng/mL LPS (positive control), or rL-TGF-β2 at concentrations of 100, 500, or 1000 ng/mL. Then, 200 μL of the leukocyte suspension was added to the upper compartment of each well. The plates were incubated at 18 °C for 3 h. Post-incubation, the polycarbonate membranes were carefully removed, and cells on the upper membrane surface were gently removed with a cotton swab. Membranes were washed twice with PBS. Membranes were fixed by adding 500 μL of methanol to each well and incubated at room temperature for 30 min. Following air-drying for 10 min, membranes were stained with 500 μL of 0.1 % crystal violet solution at 37 °C for 15 min. After staining, membranes were washed 2–3 times with PBS to remove excess dye. Membranes were dried, and images were acquired for analysis.

To evaluate the effect of rL-TGF-β2 on activated leukocyte migration, leukocytes were activated by incubation in low-serum medium containing 0.5 μg/mL LPS for 2 h. Post-activation, adherent cells were detached by incubation with 0.2 % EDTA solution at 4 °C for 5 min. Cells were washed, resuspended in low-serum medium, and subjected to the migration assay as described for quiescent leukocytes.

Migrated cells were visualized and imaged using an inverted microscope (CKX53, Olympus) at 200 × magnification. The experiment was independently replicated four times, with eight randomly selected fields imaged.

2.3. Recombinant L-TGF-β2 stimulation and real-time quantitative PCR

For the recombinant L-TGF-β2 stimulation assay, leukocytes from unimmunized or LPS-immunized lampreys were seeded into 12-well culture plates with low-serum medium. Leukocytes were treated with 1 μg/mL rL-TGF-β2 and incubated at 18 °C for 1, 6, or 24 h. For the rL-TGF-β2 and cycloheximide (CHX) co-treatment assay, leukocytes were pretreated with 10 μg/mL CHX for 1 h prior to adding 1 μg/mL rL-TGF-β2, followed by incubation for 1 or 6 h. Post-treatment, leukocytes from each well were collected.

Total RNA was extracted from each sample with the SteadyPure Universal RNA Extraction Kit (Accurate Biotechnology). First-strand cDNA was synthesized with the MightyScript Plus First Strand cDNA Synthesis Master Mix (Sangon Biotech). A no-reverse-transcriptase (No-RT) control was prepared for each sample. Quantitative PCR (qPCR) was performed with the SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology), using lamprey *GAPDH* as the internal control. Ct values for the *L-LECT2*, *L-MIF*, *L-IL8*, *L-IL17*, *L-HMGB1*, and *L-GAPDH* genes were measured with the 7500 Real-Time PCR System (Applied Biosystems), with each sample analyzed in triplicate. Relative transcript levels of target genes were determined using the $2^{-\Delta\Delta Ct}$ method. To confirm amplification specificity, a melt curve analysis was performed for each qPCR reaction. Primer sequences for qPCR are provided in Supplementary Table 1.

2.4. Immunoblot analysis

Tissue samples from unimmunized or LPS-immunized lampreys were homogenized in 200 μL of RIPA lysis buffer [50 mM Tris-HCl (pH 7.5), 1 % (v/v) Triton X-100, 0.25 % (w/v) sodium deoxycholate, 0.1 % (w/v) sodium dodecyl sulfate (SDS), 0.15 M NaCl, 1 mM EDTA, and a protease inhibitor cocktail (Sigma-Aldrich)]. Homogenates were centrifuged at 20,000×*g* for 20 min, and total protein concentration in the supernatant was determined with the Bradford assay (Bio-Rad), using fatty acid-free bovine serum albumin as a standard. Equal protein amounts (20 μg per sample) were separated by SDS-PAGE and transferred onto PVDF membranes.

Membranes were sequentially probed with primary antibodies against human or mouse TGFBR1, TGFBR2, and GAPDH, chosen for their high sequence homology with lamprey orthologs: (1) rabbit polyclonal anti-TGFBR1 (ab31013, Abcam); (2) rabbit polyclonal anti-TGFBR2 (AP17322b, Abcepta); (3) mouse monoclonal anti-GAPDH (2B5) (A01020, Abbkine). Antibody specificity was validated by comparing immunoblots of lamprey leukocyte and human MCF-7 cell lysates incubated with primary antibodies against TGFBR1, TGFBR2, and GAPDH or their corresponding isotype controls, with MCF-7 serving as a positive control (Supplementary Fig. 1). Following overnight incubation with primary antibodies at 4 °C, membranes were incubated with secondary antibodies: HRP-conjugated goat anti-rabbit IgG (H + L) (SA00001-2, Proteintech) or HRP-conjugated goat anti-mouse IgG (H +

L) (SA00001-1, Proteintech). Between probing steps, membranes were stripped with elution buffer (Meilunbio), thoroughly washed, and re-blocked. Immunoreactive bands were detected with Pierce ECL Plus Substrate (Thermo Fisher Scientific). Band optical densities were quantified using ImageJ software, with relative expression levels of L-TGFBR1 and L-TGFBR2 normalized to L-GAPDH as an internal control. Each treatment comprised three biological replicates.

2.5. Statistical analysis

GraphPad Prism 8 (GraphPad Software Inc., USA) was utilized for all statistical analyses. Data are presented as means ± SEM. A p-value <0.05 was considered statistically significant ($^*p < 0.05$, $^{**}p < 0.01$).

3. Results and discussion

Employing a Boyden chamber assay, we examined the effect of recombinant L-TGF-β2 on peripheral blood leukocyte chemotaxis in lampreys. Results revealed a significant, dose-dependent biphasic effect of L-TGF-β2 on the migration of quiescent and LPS-activated leukocytes. In adult Japanese lampreys (*Lampetra japonica*), increasing L-TGF-β2 concentrations (100–1000 ng/mL) significantly augmented quiescent leukocyte migration (Fig. 1A). Specifically, leukocyte migration was 2.21-fold higher with 100 ng/mL L-TGF-β2 treatment compared to the control and 3.27-fold higher at 1000 ng/mL. Notably, the concentrations employed reflect the total protein content of prokaryotically expressed recombinant L-TGF-β2, with the biologically active dimeric form comprising only a minor fraction. Consequently, higher nominal concentrations were necessary compared to commercially available human or mouse TGF-β2 recombinant proteins expressed in eukaryotic systems, which are predominantly active dimers, consistent with prior studies (Cai et al., 2010; Liu et al., 2022; Wang et al., 2014). These findings suggest that L-TGF-β2 effectively induces leukocyte migration under quiescent conditions, highlighting its role in facilitating immune cell localization and recruitment in non-inflammatory states.

In contrast, L-TGF-β2 inhibited leukocyte migration in LPS-activated cells, with the effect intensifying as L-TGF-β2 concentration increased (Fig. 1B). At 1000 ng/mL L-TGF-β2, migration inhibition reached 76.5 %. This indicates that L-TGF-β2 not only promotes leukocyte migration in quiescent states but also effectively curbs excessive migration during inflammation, underscoring its dual regulatory role.

Our findings align with the established roles of TGF-β in higher vertebrates, where comparable effects have been noted in bony fish (Cai et al., 2010). This further implies that the evolutionary conservation of TGF-β signaling pathways in regulating immune cell migration originated in early vertebrates. Although the TGF-β homolog in amphioxus (*Cephalochordata*), a more primitive chordate than lampreys, bidirectionally regulates mouse macrophage migration *in vitro*, evidence that amphioxus TGF-β modulates its own leukocyte migration remains absent (Wang et al., 2014). Thus, our study demonstrates that the context-dependent regulation of immune cell chemotaxis by TGF-β signaling traces back to jawless vertebrates, such as lampreys.

To further elucidate the role of TGF-β signaling in modulating inflammatory mediator expression during the lamprey innate immune response, we performed quantitative PCR to evaluate transcript levels of key pro-inflammatory cytokines in L-TGF-β2-treated leukocytes. These comprised *L-LECT2*, *L-MIF*, *L-IL8*, *L-IL17*, and *L-HMGB1*.

L-LECT2 (leukocyte cell-derived chemotaxin 2) is a chemokine that enhances lamprey leukocyte migration and phagocytosis *in vitro* (Wang et al., 2018). L-MIF (macrophage migration inhibitory factor), a pro-inflammatory cytokine secreted by T lymphocytes and macrophages in response to inflammatory stimuli, has a cloned lamprey homolog (Sato et al., 2003). L-IL17, an evolutionarily conserved pro-inflammatory cytokine, plays a pivotal role in innate immune responses by inducing downstream inflammatory mediators, including IL-8, IL-1β, and TNF-α. Lamprey homologs of IL-8 (L-IL8) and IL-17

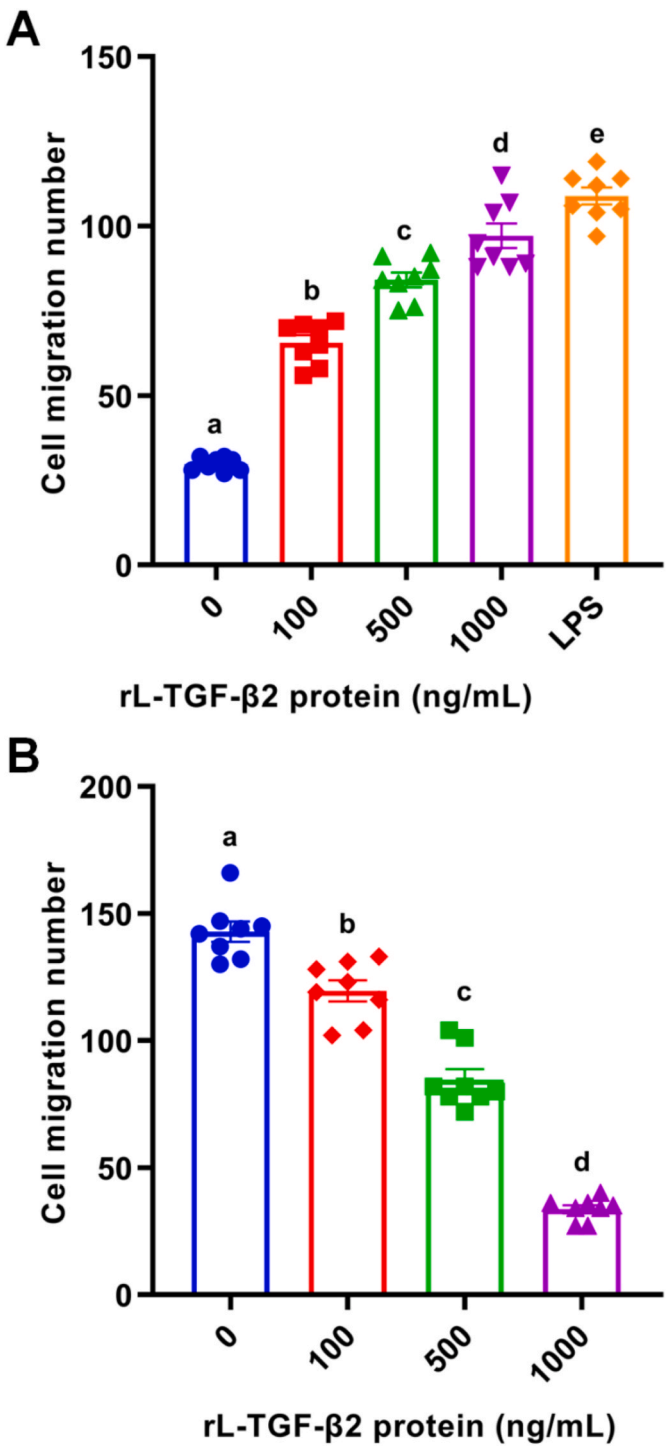

Fig. 1. Effect of recombinant L-TGF-β2 on quiescent and activated leukocyte migration in lampreys, evaluated via Boyden chamber assay. Leukocytes were treated with varying doses of recombinant L-TGF-β2 protein, using LPS as a positive control and PBS as a negative control. (A) Effect of rL-TGF-β2 on quiescent lamprey leukocyte migration. (B) Effect of rL-TGF-β2 on LPS-activated lamprey leukocyte migration. Data are presented as means ± SEM of cell counts from eight randomly selected fields across four independent experiments. Representative micrographs of lamprey leukocytes migrating into the lower chamber are provided in Supplementary Fig. 2. Statistical analyses were conducted using one-way ANOVA with Tukey's multiple comparisons test. Distinct letters above bars denote statistically significant differences between groups ($p < 0.05$).

(L-IL17) have been identified (Han et al., 2015; Najakshin et al., 1999). Extracellular L-HMGB1 (high-mobility group box 1 protein), a potent pro-inflammatory mediator, contributes to activating key molecules in the lamprey inflammatory signaling pathway post-LPS stimulation (Li et al., 2019). Prior studies indicate that transcript levels of these inflammatory mediators are significantly elevated in lamprey SMB (supraneural myeloid body) cells and leukocytes following LPS stimulation (Li et al., 2019; Wang et al., 2019).

In quiescent leukocytes, L-TGF-β2 significantly increased transcript levels of all tested pro-inflammatory cytokines (Fig. 2A–E). Notably, *L-MIF* and *L-LECT2* transcript levels peaked within 1 h of treatment, while *L-IL8*, *L-IL17*, and *L-HMGB1* reached maximal expression at 6 h, followed by a gradual decline. This early activation suggests that TGF-β signaling may directly regulate transcript levels of these genes to initiate a pro-inflammatory response.

To ascertain whether this upregulation results from direct transcriptional regulation, we co-treated quiescent leukocytes with L-TGF-β2 and cycloheximide (CHX), a protein synthesis inhibitor, at 1- and 6-h time points (Fig. 2K–O). With CHX, *L-LECT2* expression was significantly elevated at 1 h and further increased by 6 h (Fig. 2K), suggesting that L-TGF-β2 directly activates *L-LECT2* independently of new transcription factor synthesis. This is consistent with the canonical TGF-β/Smad signaling pathway, wherein Smad proteins directly regulate transcription. In contrast, *L-MIF*, *L-IL8*, and *L-IL17* exhibited no significant upregulation at 1 h but were markedly elevated by 6 h (Fig. 2L–N). This pattern indicates that in quiescent leukocytes, early (1-h) induction of these genes depends on *de novo* protein synthesis (indirect regulation), whereas later (6-h) upregulation may involve protein-synthesis-independent mechanisms, such as mRNA stabilization or chromatin remodeling. Conversely, *L-HMGB1* transcription remained unaltered at

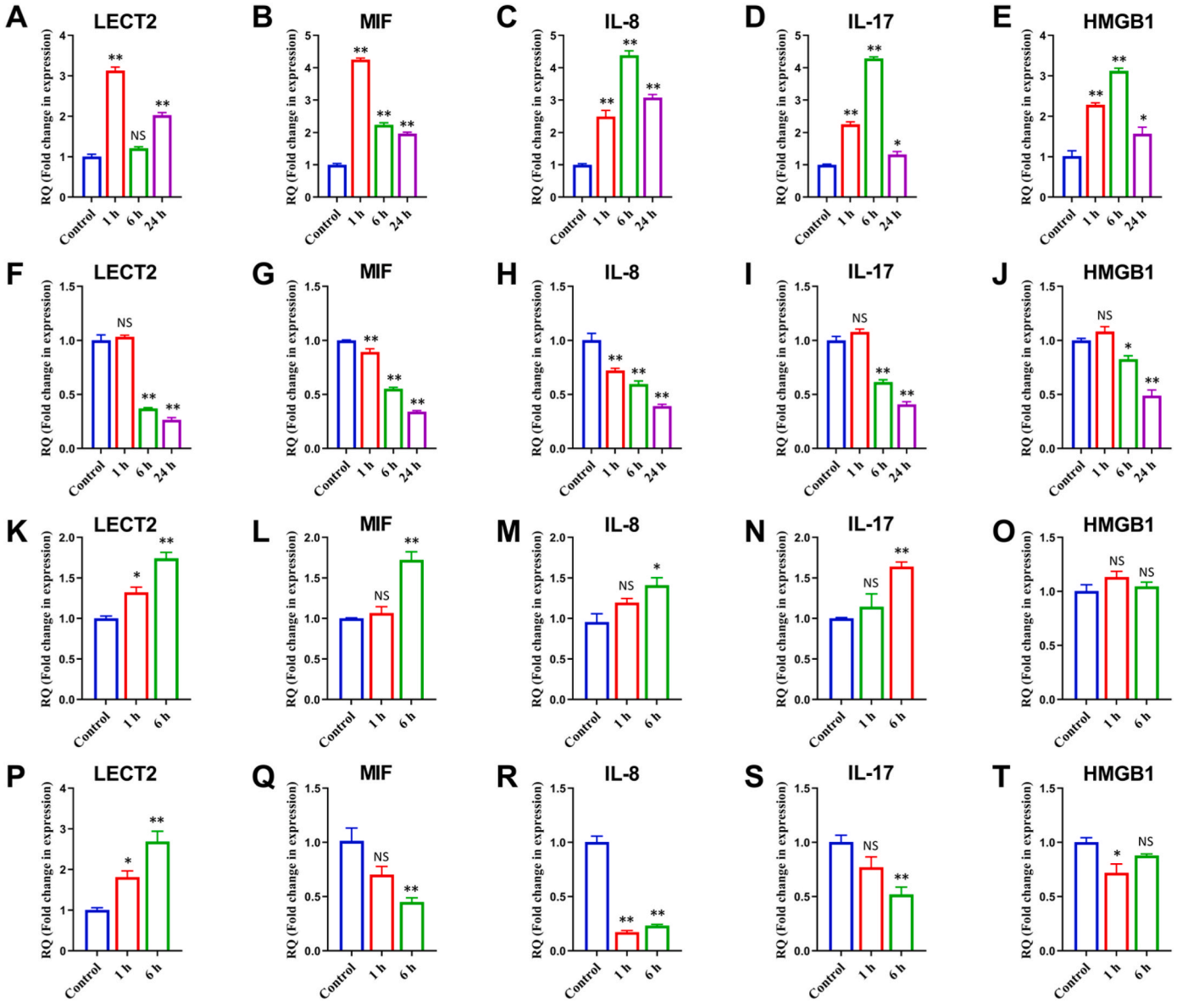

Fig. 2. Effects of rL-TGF-β2 on pro-inflammatory cytokine transcript levels in lamprey leukocytes. (A–E) Transcript levels of *L-LECT2*, *L-MIF*, *L-IL8*, *L-IL17*, and *L-HMGB1* in quiescent leukocytes post-treatment with rL-TGF-β2 for 1, 6, or 24 h. **(F–J)** Transcript levels of the same cytokines in LPS-activated leukocytes treated with rL-TGF-β2 for 1, 6, or 24 h. Relative transcript levels were quantified via real-time PCR and normalized to the control group. **(K–O)** Effects of cycloheximide (CHX) co-treatment on rL-TGF-β2-mediated cytokine expression in quiescent leukocytes, assessed at 1- and 6-h post-treatment. **(P–T)** Effects of CHX co-treatment on rL-TGF-β2-mediated cytokine expression in LPS-activated leukocytes, assessed at 1- and 6-h post-treatment. Relative transcript levels were quantified via real-time PCR and normalized to the CHX-treated control group. Data are presented as means ± SEM from three independent experiments. Statistical analyses were conducted using one-way ANOVA with Dunnett's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, NS: not significant.

both time points with CHX treatment (Fig. 2O), suggesting that its activation by L-TGF-β2 wholly depends on indirect mechanisms involving newly synthesized transcription factors. The early upregulation of cytokines like *L-LECT2* and *L-MIF* in quiescent leukocytes suggests that L-TGF-β2 is not simply a pro-inflammatory response activator. Rather, its role reflects the complexity of the signaling network mediating inflammation initiation, as substantiated by the direct and indirect regulatory mechanisms elucidated through CHX co-treatment experiments.

In LPS-activated leukocytes, L-TGF-β2 exhibited a marked inhibitory effect (Fig. 2F–J). At 1 h, *L-MIF* and *L-IL8* expression decreased slightly, with all tested cytokines significantly suppressed by 6 h. This suppression peaked at 24 h, underscoring L-TGF-β2's pivotal role in mitigating excessive inflammation.

To investigate regulatory mechanisms in activated cells, we co-treated LPS-stimulated leukocytes with CHX and L-TGF-β2 (Fig. 2P–T). *L-IL8* expression was significantly reduced at both 1 and 6 h (Fig. 2R), consistent with L-TGF-β2 treatment alone, confirming direct transcriptional suppression by L-TGF-β2. However, *L-MIF* and *L-IL17* exhibited no significant reduction at 1 h, with downregulation apparent only at 6 h (Fig. 2Q and S), indicating early indirect regulation succeeded by a protein-synthesis-independent mechanism. Notably, *L-LECT2* transcription increased at both 1 and 6 h with CHX co-treatment (Fig. 2P), while *L-HMGB1* was downregulated at 1 h but unaltered at 6 h (Fig. 2T). These results, diverging from trends observed with L-TGF-β2 alone in activated cells (Fig. 2F and J), suggest that in immune-activated conditions, L-TGF-β2 regulates *L-LECT2* and *L-HMGB1* via indirect mechanisms involving additional transcriptional regulators.

These findings highlight the dual role of TGF-β signaling in lampreys, analogous to higher vertebrates, promoting inflammation in quiescent states and suppressing it during resolution. The direct regulation of *L-LECT2* and *L-IL8* aligns with Smad-mediated mechanisms, whereas the indirect control of *L-MIF*, *L-IL17*, and *L-HMGB1* indicates a complex regulatory network. Unlike mammals, where TGF-β typically upregulates *IL-8* and *IL-17* through *de novo* transcription factor synthesis (Lu and Dong, 2006; Yang et al., 2008) and no direct induction of *LECT2* (Zhao et al., 2022) or *MIF* (Takahashi et al., 1998) has been documented, lampreys demonstrate direct, context-specific regulation of certain cytokines, indicating specialized immune homeostasis mechanisms that reflect both conservation and divergence in TGF-β signaling pathways, providing fresh insights into vertebrate immune evolution.

In higher vertebrates, the regulatory effects of TGF-β signaling are tightly linked to the expression levels of its two primary cell surface receptors: TGF-β type I receptor (TGFBR1) and TGF-β type II receptor (TGFBR2). To further elucidate the mechanisms underlying the context-dependent regulation of TGF-β signaling in lamprey inflammation, we cloned the lamprey homologs of TGFBR1 and TGFBR2 and analyzed their expression in key immune tissues following LPS-induced inflammation using Western blotting. Our results revealed that LPS treatment significantly upregulated L-TGFBR1 and L-TGFBR2 expression in lamprey leukocytes, suggesting enhanced TGF-β signaling during inflammation (Supplementary Fig. 3A–D).

Conversely, in the gill tissues of LPS-treated lampreys, L-TGFBR1 expression remained unchanged, whereas L-TGFBR2 expression was significantly downregulated (Supplementary Fig. 3E–H). Furthermore, no significant differences were observed in L-TGFBR1 or L-TGFBR2 expression levels in the supraneural body tissues of LPS-treated lampreys compared to controls (Supplementary Fig. 3I–L). These tissue-specific variations indicate that TGF-β receptor expression in distinct immune microenvironments may be modulated by inflammatory mediators, thereby influencing TGF-β signaling pathway activation. This observation aligns with findings in higher vertebrates, underscoring that differential TGF-β receptor expression is fundamental to the diverse immune-regulatory functions of TGF-β in lampreys.

In conclusion, this study demonstrates that L-TGF-β2 in lampreys exerts a dual role in immune responses by modulating leukocyte migration and pro-inflammatory cytokine transcription, thus maintaining immune homeostasis. This biphasic regulatory function parallels that of TGF-β in higher vertebrates, underscoring the evolutionary conservation of the TGF-β signaling pathway across vertebrates and suggesting that TGF-β ranks among the earliest immune cytokines in vertebrates. Although the regulatory mechanisms of the TGF-β signaling pathway in lampreys diverge slightly from those in higher vertebrates, its pivotal role in sustaining immune homeostasis offers novel insights into vertebrate immune system evolution. Future studies could further investigate the distinct mechanisms of TGF-β signaling in lampreys, illuminating the diversity and evolutionary conservation of TGF-β in immune regulation.

CRediT authorship contribution statement

Hao Wang: Writing – review & editing, Writing – original draft, Project administration, Investigation, Funding acquisition. **Junfu Guo:** Writing – original draft, Investigation. **Xuanyi Chen:** Writing – original draft, Investigation. **Siqi Liu:** Writing – original draft, Investigation. **Wenna Li:** Writing – original draft, Investigation. **Lu Yang:** Visualization, Validation. **Jianmiao Wang:** Visualization, Validation. **Yinglun Han:** Writing – review & editing, Supervision, Funding acquisition.

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Declaration of competing interest

All authors declare no conflicts of interest.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2025.105372.

Data availability

Data will be made available on request.

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