Allergy

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Allergen Protease Act d 1 Induces TLR4-Dependent Inflammation via Ectodomain Cleavage

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Received: 6 August 2025 | **Revised:** 1 July 2026 | **Accepted:** 16 July 2026

To the Editor,

Recent findings suggest that allergen proteases activate immune responses through mechanisms closely related to their proteolytic activity, a key factor influencing the course of the immune response [1]. While in atopic individuals, protease allergens drive sensitization to their own epitopes through IgE-dependent mechanisms, their ability to trigger innate inflammatory pathways positions them as primary initiators of inflammation [1]. Actinidin (Act d 1), a cysteine protease and a major kiwifruit allergen, exhibits high gastrointestinal stability and retains its enzymatic activity, which contributes to its effects on epithelial and immune cells [2]. Its impact on epithelial integrity is well studied, including increased epithelial permeability, degradation of tight junctions, and upregulation of cytokines [2]. While no specific receptor has been identified for actinidin, allergen proteases other than cysteine proteases are known to activate protease-activated receptors (PARs) [3, 4]. In this study, we reveal the crucial role of the TLR4 receptor in actinidin-induced pro-inflammatory response in immune cells.

Firstly, we confirmed that Act d 1 induces a potent pro-inflammatory response in mouse bone marrow-derived macrophages (BMDMs), characterized by concentration-dependent production of IL-6 and TNFα (Figure S1) without affecting cell viability (Figure S2). This is accompanied by NF-κB activation, as observed in RAW-Blue macrophages (Figure S1). TLR4 activation is involved in diverse pathological conditions, including allergic inflammation triggered by cleavage products of protease allergens [1], as well as direct activation induced by contact allergens [5]. Given how both NF-κB activation and pro-inflammatory cytokine production can be hallmarks of TLR4 signaling [3], we tested the involvement of TLR4 using two model systems: TLR4 knockout BMDMs and HEK293 cells transfected with TLR4. In TLR4-deficient cells, Act d 1-induced cytokine production (Figure 1A,B,E) and NF-κB activation (Figure 1C) were significantly affected, confirming TLR4-dependence. Production of TNFα was only partially reduced (Figure 1A), indicating involvement of additional pathways and receptors. Act d 1 also activated the IFNβ promoter, proving stimulation of both MyD88- and TRIF-dependent TLR4 pathways (Figure 1D). TLR4 canonical activator LPS (in complex with MD2) binds directly to TLR4 [3, 6]. While LPS cannot activate TLR4 mutant F463A, we found that the mutated LPS-insensitive receptor retained responsiveness to Act d 1 (Figure 1F,G), implying an unconventional mechanism of action, different from classical ligand-receptor interaction.

To investigate the importance of proteolytic activity, we used heat-inactivated and E64d-inhibited Act d 1 with reduced enzymatic activity (Figure S2), which led to significantly reduced NF-κB activation (Figure 2A), demonstrating that the enzymatic function of Act d 1 is essential for triggering TLR4-mediated signaling. Furthermore, western blotting showed a notable loss of full-length Myc-tagged TLR4 upon Act d 1 treatment (Figure 2B), mitigated in the presence of E64d (Figure 2C), while such an effect could not be observed upon LPS stimulation (Figure S2). Degradation of TLR4 by Act d 1 was observed in vitro using recombinant insect cell-produced His-tagged TLR4 (icTLR4), detecting a putative cleavage fragment at around 30 kDa (Figure 2D), as well as reduction of full-length TLR4 (Figure 2H and S3). In human cell-produced recombinant TLR4 (hcTLR4) distinct fragments were observed

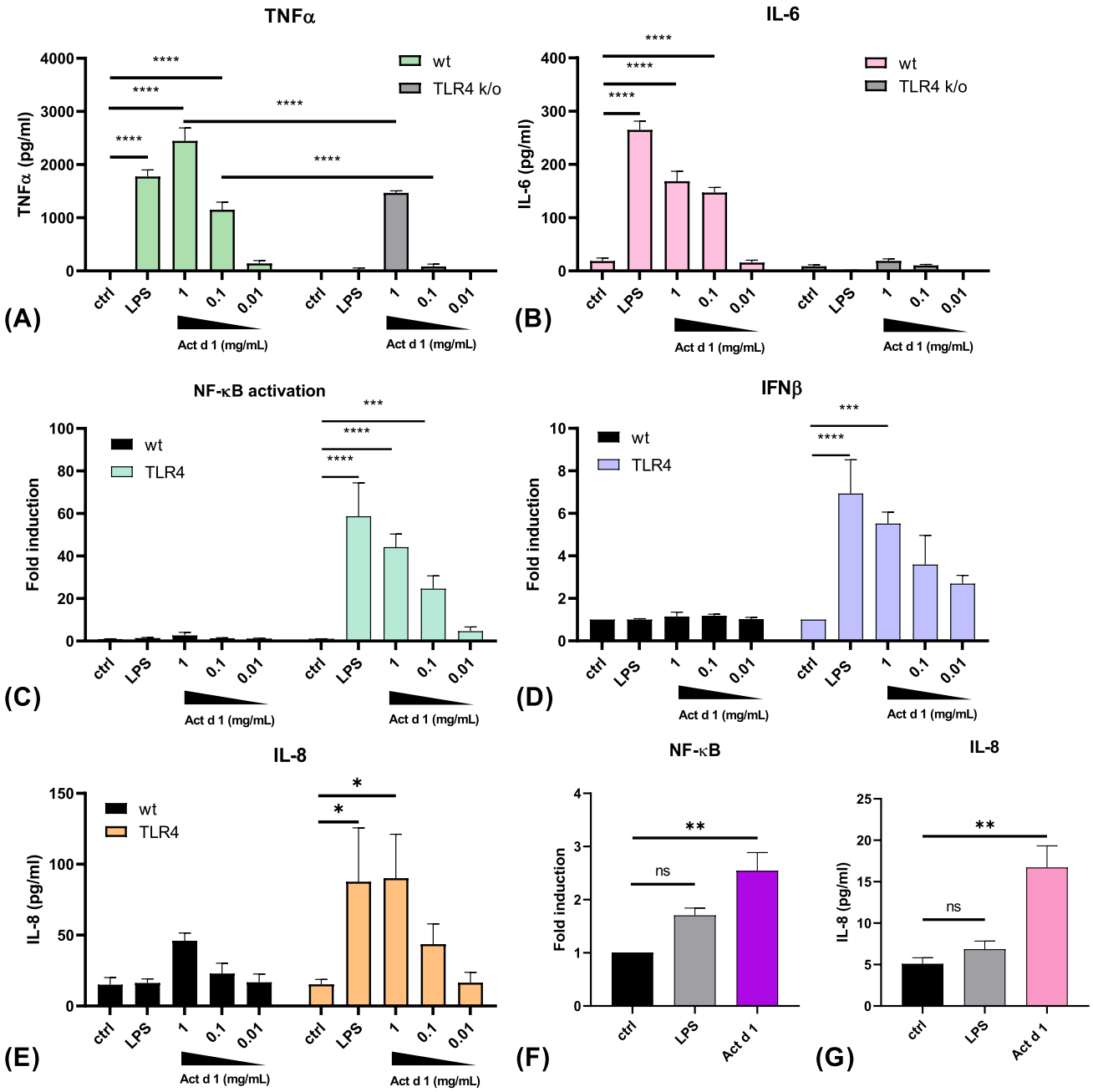

FIGURE 1 | TLR4-dependent response to Act d 1. Mouse (A) TNFα and (B) IL-6 secretion by wild-type and TLR4 k/o primary mBMDMs in response to Act d 1 and LPS (1 μg/mL). (C) NF-κB, (D) IFNβ promotor activation, (E) human IL-8 production, after treatment with LPS (100 ng/mL) and Act d 1, in HEK293 cells transfected with TLR4 or empty plasmid (pcDNA 3.0). (F) NF-κB activation and (G) IL-8 production induced by Act d 1 (1 mg/mL) and LPS (100 ng/mL) in HEK293 cells transfected with TLR4 F463A mutant. Results are presented as mean ± SEM, $n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

at approximately 16 kDa and below 6.5 kDa (Figure 2G), accompanied by a reduction of full-length TLR4 (Figure 2H and S3), indicating cleavage and receptor fragmentation, with differences in fragment size and intensity, presumably due to differences in posttranslational modifications of the two expression systems used. Act d 1 failed to enhance NF-κB activation (Figure 2E) and IL-8 production (Figure 2F) in a constitutively active TLR4 Δ1–563 construct lacking most of the ectodomain, suggesting that Act d 1 causes the cleavage of TLR4 ectodomain, thereby removing the region that maintains TLR4 in an inactive state in the absence of its ligand [6].

In conclusion, our findings provide evidence that the cysteine protease allergen Act d 1 elicits a pro-inflammatory response through TLR4-dependent mechanisms. Importantly, this response is linked to the proteolytic activity of Act d 1, which appears to activate TLR4 not by classical ligand binding, but rather by causing degradation of the TLR4 ectodomain. This alternative mechanism of receptor activation broadens the understanding of how protease allergens can engage innate immune receptors and initiate inflammation and adds growing recognition of the role of enzymatic activity in allergen immunogenicity [1].

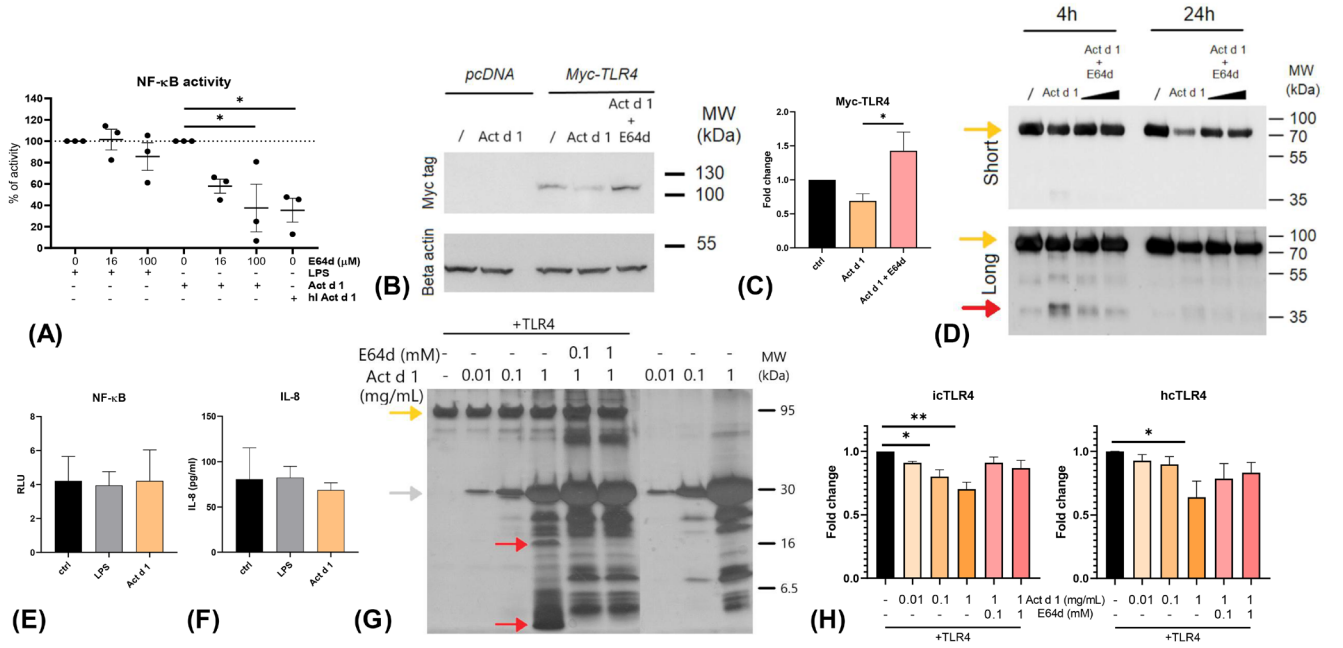

FIGURE 2 | Role of proteolytic activity in TLR4 activation. (A) Percentage of NF-κB activity induced by LPS, preactivated Act d 1, heat-inactivated (hI) and E64d-inhibited Act d 1 in HEK293 cells transfected with TLR4. (B) Expression of human TLR4 construct tagged on its N-terminal domain using c-Myc tag (Myc-hTLR4) after treatment with preactivated and E64d-inhibited Act d 1, and (C) densitometric analysis of the expression levels. (D) In vitro digestion of icTLR4 ectodomain after 4 and 24 h of incubation with preactivated and E64d-inhibited (100 μM and 1 mM) Act d 1 using western blot detection under short and long exposure times. (E) NF-κB activation and (F) IL-8 secretion following treatment in HEK293 cells transfected with a deletion variant of human TLR4 Δ1–563. (G) In vitro digestion of hcTLR4 ectodomain detected by silver staining detection (long development time). (H) Densitometric analysis of full-length TLR4 bands detected by silver staining following in vitro digestion with Act d 1. Arrows: Yellow-intact TLR4, gray-Act d 1, red-putative TLR4 fragments. Results are presented as mean ± SEM, $n = 3$; *$p < 0.05$, **$p < 0.01$.

Acknowledgments

The authors are grateful to Dr. Mateja Manček Keber for valuable suggestions and Klementina Podgoršek for technical assistance. This study contributes to Good Health and Well-Being (SDG 3) by elucidating how the kiwifruit allergen Act d 1 triggers TLR4-dependent innate immune activation, advancing understanding relevant to improved risk assessment and prevention of allergic diseases.

Funding

This research was supported by the Federation of European Biochemical Societies (FEBS) Short-Term Fellowship for the year 2025, the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract number: 451-03-33/2026-03/200168), and Slovenian Research and Innovation Agency (ARIS) project grant J3-60056 and ARIS research core (programme) funding P4-176.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Secretion of pro-inflammatory cytokines (A) TNFα and (B) IL-6 in mouse BMDM supernatants after 4 h of treatment with LPS (1 μg/mL) or Act d 1. (C) NF-κB activation in mouse reporter RAW-Blue macrophages after 24 h of treatment with LPS (100 ng/mL) and Act d 1. Results are presented as mean ± SEM, $n = 3$; *p value < 0.05, ** p value < 0.01, *** p value < 0.001, and **** p value < 0.0001. **Figure S2:** (A) LDH assay was used to measure cell damage on BMDMs after 4 h of treatment with LPS (1 μg/mL) without and with NLRP3 inflammasome-pyroptosis activator nigericin (10 μM) for 1 h as a positive control, and Act d 1 for 4 h. LDH activity was measured in cell supernatants and % of LDH release was calculated. (B) Cell viability was analyzed via PI uptake assessment after 24 h of treatment with Act d 1

and LPS (1 μg/mL) following incubation with PI solution (3.3 μg/mL) for 30 min. PI fluorescence was measured and % of uptake was calculated. For both assays, cells treated with lysis buffer were used as 100% positive control. (C) Assay using a synthetic substrate (BAPNA) was used to analyze the enzymatic activity of preactivated, heat inactivated and E64d-inhibited Act d 1. (D) Expression of human Myc-TLR4 after LPS stimulation in the presence or absence of inhibitor E64d (100 μM) along with expression of beta actin was analyzed on western blot. Results are presented as mean ± SEM, $n = 3$; ** p value < 0.01, *** p value < 0.001 and **** p value < 0.0001. **Figure S3:** In vitro digestion of recombinant TLR4 ectodomain. Analysis of hcTLR4 ectodomain using silver staining detection under (A) short development time and icTLR4 ectodomain using silver stain detection under (B) short and (C) long development time. Digestion conditions included 4 h-incubation of the proteins with preactivated Act d 1 (0.01, 0.1 or 1 mg/mL) and E64d-inhibited Act d 1 (1 mg/mL in the presence of 100 μM or 1 mM E64d). **Data S1:** Supporting Information