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Plant Science LETTER

The Innovation Life

Plant-derived RSV pre-F with ER retention exhibits enhanced macrophages uptake and immune effects

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Received: June 8, 2026; Accepted: August 25, 2026; Published Online: September 2, 2026; https://doi.org/10.59717/j.xinn-life.2026.100248

Citation: Diao H.-P., Wang J.-R., Zhang J.-F., et al. (2026). Plant-derived RSV pre-F with ER retention exhibits enhanced macrophages uptake and immune effects. *The Innovation Life* **4**:100248.

Dear Editors,

Plant molecular farming (PMF), which uses plants instead of mammalian cells to produce recombinant proteins, has matured over the past four decades and enabled the production of vaccines, antibodies, and therapeutic enzymes.[1,2] Several plant-derived recombinant vaccines have recently received regulatory approval, demonstrating the translational potential of this technology.[3,4] Nevertheless, PMF has been regarded primarily as a manufacturing platform, valued for its scalability, cost-effectiveness, and freedom from animal-derived pathogens.[5] Whether plant expression systems also impart intrinsic molecular features that influence the biological functions of recombinant antigens remains insufficiently understood.

Protein glycosylation differs substantially between plant and mammalian expression systems and plays critical roles in antigen stability, receptor interactions, cellular uptake, and immune recognition.[6] Importantly, the glycosylation profiles of recombinant proteins are strongly influenced by their intracellular trafficking routes. In PMF, ER retention is frequently employed to enhance protein accumulation and preserve high-mannose N-glycans,[1] whereas mammalian expression systems typically rely on the secretory pathway, during which proteins undergo extensive Golgi-mediated glycan processing and acquire more complex N-glycan structures. These differences raise the possibility that host-specific processing environments may influence antigen function beyond production efficiency. However, whether and how such processing affects antigen-immune interactions remains unclear.

In this study, we employed the RSV pre-fusion F protein variant PR-DM (N67I, S215P) [7] as a model antigen to examine how plant-specific post-translational modifications influence antigen immunogenicity. ER-retained Pre-F trimers were expressed in the PPO-knockout *N. benthamiana* chassis (*ppoa;ppob#3*) [8] for efficient purification and subsequently purified by Ni^{2+}-NTA affinity and size-exclusion chromatography. To enable a comprehensive comparative analysis of *N.b.*-F, Pre-F was also produced in mammalian HEK293 cells, yielding HEK293-F. As the HEK293 expression system is secretion-based, the recombinant protein utilized a mammalian signal peptide distinct from that used in the plant expression system and lacked a C-terminal ER retention motif. Nevertheless, the core coding sequence of the Pre-F antigen remained identical between the two expression platforms. Notably, neither signal peptide contained predicted N-glycosylation sites. The HEK293-F was also subsequently purified using a two-step procedure comprising Ni^{2+}-NTA affinity chromatography followed by size-exclusion chromatography. Recombinant Pre-F proteins from both expression platforms were successfully prepared for subsequent experiments (Figure 1A).

First, we verified the pre-fusion conformation of Pre-F derived from the two expression platforms. Purified HEK293-F and *N.b.*-F were analyzed by SDS-PAGE followed by Western blotting using site Ø and site Ⅱ specific antibodies. Both antibodies produced positive signals, confirming the presence of the expected antigenic sites (Figure 1B). The site Ø epitope (N63 - A74 and D200 - S213) is exclusively present in the pre-fusion conformation, whereas site Ⅱ epitope (S255 - N276) is retained in both pre-fusion and post-fusion conformations of the F protein (Figure 1C). To further confirm the presence of the site Ø epitope in native HEK293-F and *N.b.*-F, a His-tag pull-down assay was performed (Figures 1D-E). The results demonstrated that native proteins from both expression platforms can specifically interact with the site Ø specific antibody. Collectively, these findings suggest that HEK293-F and *N.b.*-F share comparable pre-fusion antigenic features. With no further evidence for substantial differences in antigenic conformation between the two proteins, subsequent analyses were directed toward investigating differences in glycosylation and their functional implications.

To delineate the glycosylation profiles of HEK293-F and *N.b.*-F, we performed enzymatic deglycosylation assays using PNGase F and Endo H. The N-linked glycans of *N.b.*-F were efficiently removed by both PNGase F and Endo H. In contrast, while HEK293-F was fully sensitive to PNGase F, it displayed pronounced resistance to Endo H digestion (Figures 1F-G). Given that Endo H specifically cleaves high-mannose N-glycans but not complex N-glycans, the differential sensitivity observed here indicates that ER-retained *N.b.*-F predominantly carries high-mannose N-glycosylation, whereas HEK293-F mainly harbors complex N-glycosylation.

To further validate the results of the glycosidase digestion assays, we subjected HEK293-F and *N.b.*-F to proteolytic digestion, followed by site-specific N-glycosylation profiling using LC-MS/MS. Glycoproteomic profiling revealed distinct glycosylation patterns between the two expression platforms. In this batch, HEK293-F contained two occupied N-glycosylation sites (N2 and N453), whereas *N.b.*-F displayed three modified sites (N2, N45, and N453), all of which were consistent with the predicted NxS/T consensus motifs (Figure 1H). Despite having fewer glycosylation sites, HEK293-F exhibited greater glycan heterogeneity, with 25 distinct glycoforms identified across the two sites. In contrast, *N.b.*-F, although glycosylated at three sites, showed a more restricted glycan repertoire, with 15 glycoforms detected (Figure 1I). Classification of glycan types demonstrated that the three sites on *N.b.*-F were predominantly modified with high-mannose N-glycans, accounting for 89.5% (N2), 100% (N45), and 87.5% (N453) of total glycans, respectively. Conversely, the two glycosylation sites on HEK293-F were mainly decorated with complex N-glycans, with high-mannose structures representing only 1.28% (N2) and 3.96% (N453) (Figure 1J). Together, these results indicate that ER-targeted retention and storage in the plant system confers a clear advantage in promoting glycosylation uniformity, leading to a more homogeneous high-mannose glycan profile.

The macrophage mannose receptor (MR, CD206), a C-type lectin predominantly expressed on the surface of macrophages and dendritic cells, mediates antigen recognition and uptake by binding mannose-containing glycans.[9] As *N.b.*-F contains predominantly high-mannose N-glycans, we reasoned that it might be more efficiently recognized and internalized by macrophages than HEK293-F. To test this hypothesis, macrophages were incubated with HEK293-F or *N.b.*-F, and antigen uptake was quantified in vitro. Macrophages were first polarized by IL-4-induced alternative activation,[10] and immunofluorescence staining confirmed a marked upregulation of CD206 expression, establishing a suitable model for receptor-mediated uptake analysis. HEK293-F and *N.b.*-F (100 μg each) were covalently labeled with Cy5 and incubated with IL-4-induced M2-polarized macrophages. Antigen internalization was then assessed by laser scanning confocal microscopy (Figure 1K). Compared with HEK293-F, *N.b.*-F exhibited markedly stronger intracellular fluorescence and a broader cytoplasmic distribution, indicating significantly enhanced uptake by macrophages (Figure 1L). Consistently, flow cytometry analysis further confirmed that Cy5-

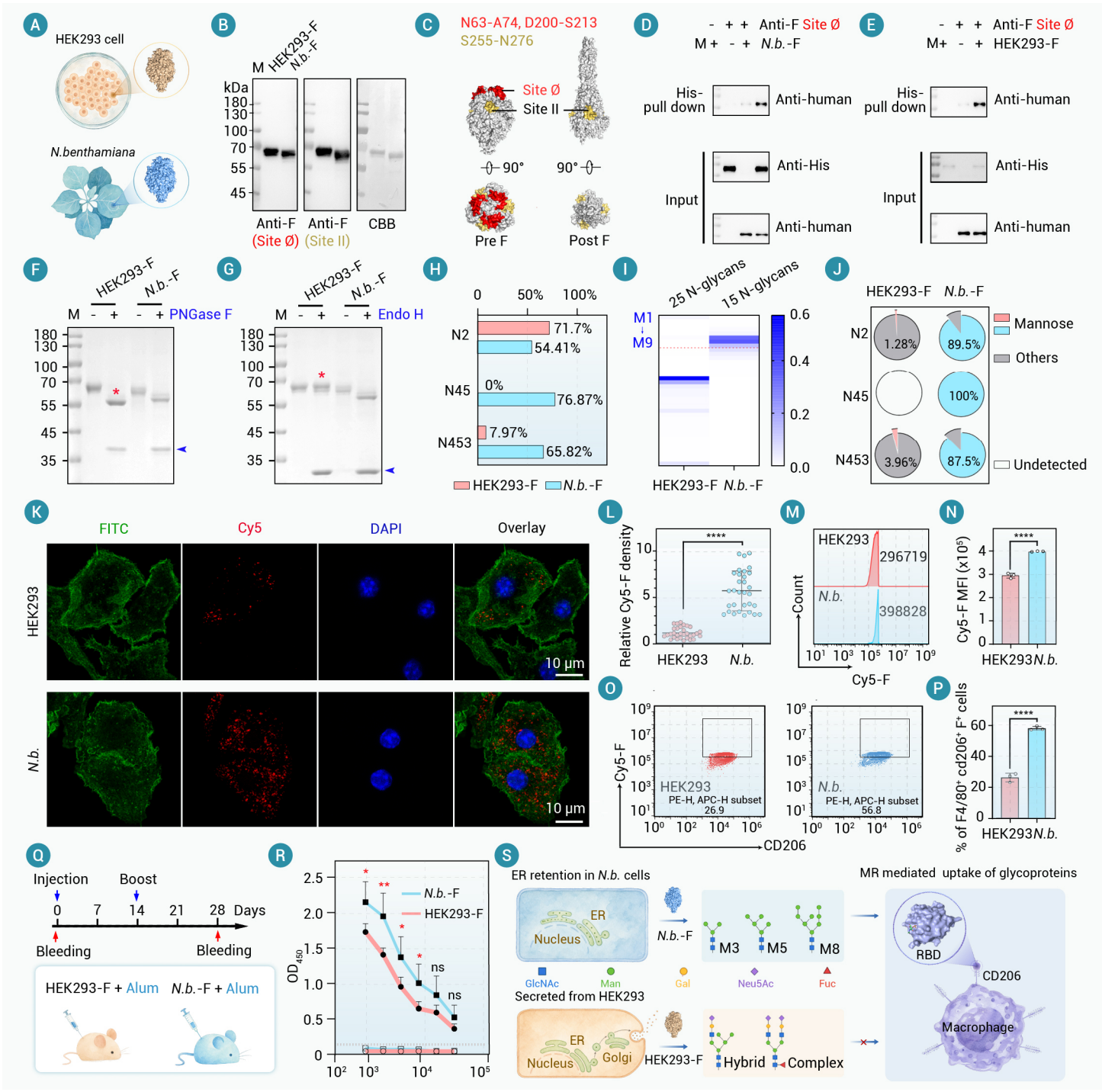

Figure 1. Plant-derived RSV Pre-F with uniform high-mannose glycosylation promotes macrophage uptake and antibody responses (A) Schematic illustration of RSV Pre-F derived from HEK293 cells (HEK293-F) and N. benthamiana (N.b.-F). (B) Purified HEK293-F and N.b.-F proteins were analyzed by SDS-PAGE followed by Western blotting using site Ø and site 2 specific antibodies. (C) Schematic illustration of RSV Pre-F protein structure and the positions of site Ø (N63-A74, D200-S213) and site 2 (S255-N276). Pull-down assays were performed to evaluate the binding of His-tagged native N.b.-F (D) and HEK293-F (E) to site Ø specific antibody. (F) N-Glycosylation analysis of purified HEK293-F and N.b.-F using PNGase F digestion. Blue arrow indicates the PNGase F binds. (G) N-Glycosylation analysis of purified HEK293-F and N.b.-F using Endo H digestion. Blue arrow indicates the Endo H binds. (H) N-Glycosylation sites and modification ratios of HEK293-F and N.b.-F identified by mass spectrometry. (I) N-Glycan types and their relative abundances identified in HEK293-F and N.b.-F by mass spectrometry. (J) Relative proportions of high-mannose and other N-glycan types at each glycosylation site of HEK293-F and N.b.-F. (K) Analysis of macrophages uptake efficiency of Cy5-labeled HEK293-F and N.b.-F. FITC was used to label the cytoskeleton, Cy5 was used to label HEK293-F and N.b.-F, and DAPI was used to stain the cell nuclei. The quantified data of the relative intracellular red fluorescence intensity are shown in panel (L). (M) Flow cytometry analysis of the uptake efficiency of Cy5-labeled HEK293-F and N.b.-F by M2 macrophages (gated on F4/80+CD206+ cells). The quantified data of the mean fluorescence intensity (MFI) of Cy5-F+ is shown in panel (N). (O) Percentage of Cy5-F+ M2 macrophages. The quantified data of the uptake ratio are shown in panel (P). (Q) Schematic illustration of the immunization protocol. Groups of five C57BL/6 mice were intramuscularly immunized with 2 μg of HEK293-F and N.b.-F, formulated with Alum, followed by a booster injection at 14-day. Mice sera were collected before and after immunization to evaluate humoral immune responses. (R) ELISA analysis of pre-immune (day 0) and 28 DPI sera from immunized mice. Dotted lines represent the positive cutoff value, calculated as the mean endpoint titer of pre-immune sera. (S) Schematic illustration of ER-retained Pre-F expressed in plant cells acquiring high-mannose glycosylation and being internalized by dendritic cells via the mannose receptor.

labeled *N.b.*-F was internalized by a higher proportion of APCs and exhibited greater intracellular fluorescence intensity compared with HEK293-F (Figures 1M-P).

To evaluate the immunogenicity of HEK293-F and *N.b.*-F in vivo, we performed intramuscular immunization in six-week-old C57BL/6 mice (Figure 1Q). Ten mice were randomly divided into two groups (n = 5 per

group) and were intramuscularly immunized with 2 μg of HEK293-F and *N.b.*-F, formulated with alum, followed by a booster injection at 14-day. Mice sera were collected before and after immunization to evaluate humoral immune responses by ELISA. The results showed that both HEK293-F and *N.b.*-F elicited robust antigen-specific antibody responses when formulated with alum. Notably, *N.b.*-F induced significantly higher antibody titers than HEK293-F on day 28 (Figure 1R), suggesting enhanced humoral immunogenicity. However, the functional activity of these antibodies and their contribution to protective immunity were not evaluated in the present study.

Overall, our work provides evidence that ER-associated glycosylation in plant expression systems may represent more than a production-related characteristic and can influence antigen-macrophage interactions. The relatively uniform high-mannose N-glycan profile generated by the plant platform was associated with increased antigen recognition and uptake by macrophages (Figure 1S). Together with the enhanced antibody responses observed in vivo, these findings suggest that plant-derived ER-associated glycosylation may contribute to the immunological properties of recombinant protein vaccines and reveal a previously underappreciated functional aspect of plant-based expression systems.

Several limitations should also be acknowledged. First, although both HEK293-F and *N.b.*-F exhibited comparable recognition by conformation-dependent antibodies, more comprehensive structural characterization was not pursued in the present study. Second, while enhanced macrophage uptake and antibody responses were associated with distinct glycosylation profiles, the causal mechanisms linking glycosylation to immune outcomes remain to be fully elucidated. Despite these limitations, our findings support the notion that host-specific glycosylation patterns may have functional consequences beyond recombinant protein production and warrant broader consideration in the design and evaluation of plant-derived vaccines.

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FUNDING AND ACKNOWLEDGMENTS

This research was supported by the Shandong Provincial Natural Science Foundation (2026HWYQ-046 and ZR2024QC068), the Agricultural Science and Technology Innovation Program (ASTIP-TRIC02 and CAAS-TRIC-2025-01), and the Science and Technology Foundation of Beijing Life Science Academy (2024200CB0110). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

S.J.S. conceived the study and wrote the manuscript. H.P.D. performed vector construction, protein purification, glycosylation analyses, ELISA assays, and other experimental work, with assistance from Y.F.G. J.R.W. carried out the in vitro macrophage experiments, with assistance from J.F.Z. The manuscript was critically reviewed and revised by I.H.H. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

ETHICAL STATEMENT AND PATIENT CONSENT

Not Applicable.

DATA AND CODE AVAILABILITY

Data are available from the corresponding author upon reasonable request.