

國立臺灣大學獸醫專業學院分子暨比較病理生物學研究所

碩士論文

Graduate Institute of Molecular and Comparative Pathobiology

School of Veterinary Medicine

National Taiwan University

Master's Thesis

探討豬隻凝集素 mKCR, Nkp44, Siglec-9, TLT2 於豬流行
性下痢病毒感染中之角色暨研發脂質奈米微粒包覆之豬流

行性下痢病毒棘蛋白 S1 結構域信使核糖核酸疫苗

Evaluation of the Role of Porcine Lectins of mKCR, Nkp44,
Siglec-9, and TLT2 in Porcine Epidemic Diarrhea Virus
Infection and Development of Lipid Nanoparticle S1-based
mRNA Vaccine Against Porcine Epidemic Diarrhea Virus

張巧邑

Qiao-Yi Chang

指導教授：張惠雯 博士

Advisor: Hui-Wen Chang, DVM, Ph.D.

中華民國 115 年 07 月

July 2026

中文摘要



豬流行性下痢病毒 (Porcine epidemic diarrhea virus, PEDV) 可引發新生仔豬嚴重的急性水樣腹瀉與嘔吐，並導致極高的致死率，對全球養豬產業造成巨大的經濟損失。然而，關於該病毒的主要功能性受體，至今在學術界仍存在諸多爭議。由於 PEDV 棘蛋白 (Spike protein) 表面覆蓋有極為複雜且高度糖基化的結構，宿主細胞表面的「糖基-凝集素交互作用」被普遍認為在病毒初期的附著與入侵過程中扮演關鍵角色。為了系統性地探討這些宿主因子，本研究透過親和力酵素免疫分析法篩選可能與 PEDV 重組棘蛋白進行交互作用的糖結合凝集素 (lectins)。本研究優先篩選出四種關鍵的候選凝集素 (mKCR、Nkp44、Siglec-9 與 TLT2)。經由生物資訊學分析進一步確認了這些候選分子在糖類辨識結構域 (CRD) 內具有高度的序列保守性，並成功鑑定出其在豬隻基因組中的特異性同源基因。為了探討這些豬源凝集素是否具備與病毒棘蛋白交互作用，本研究建構豬源凝集素 mKCR、Nkp44、Siglec-9 與 TLT2 表現載體，並將其導入具病毒感受性細胞與非感受性的細胞模型中。細胞的蛋白質表現分析證實，各候選凝集素均能成功於細胞中表達。然而，在隨後的病毒攻毒試驗中，分子檢測與病毒抗原染色結果均顯示，無論細胞是否表達這些豬源凝集素，非感受性細胞模型中皆完全無法偵測到任何病毒複製或病毒蛋白質合成的訊號。同樣地，在外源性表達這些凝集素的天然感受性細胞模型中，其內部病毒載量亦未較對照組提升。上述發現皆表明，雖然 mKCR、Nkp44、Siglec-9 與 TLT2 能與病毒棘蛋白上的糖基產生相互作用，在細胞表面提供物理性的螯合位點，但它們在感染鏈中僅扮演非感受性附著因子 (Non-permissive attachment factors) 的角色。它們可能透過多價結合的親和力將病毒顆粒錨定於細胞膜表面，充當病毒初期綁定階段角色，但本身並不具備獨立啟動膜融合或媒介具複製能力之病毒進入細胞的功能。此外，在探討病毒附著機制的同時，本研究第二部分亦針對 PEDV 高突變率與變異株流行之特性，研發出一款新型的信使核糖核酸 (mRNA) 疫苗預防策略。為克服過往實



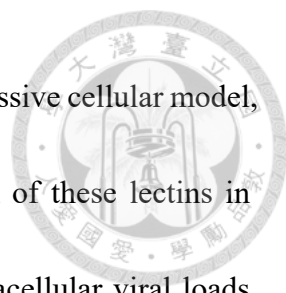
驗室使用全長棘蛋白作為抗原時所面臨的結構複雜性、細胞代謝負擔以及體內免疫保護力不彰等限制，本研究將抗原標的聚焦於包含主要中和性抗原決定之受體結合結構域的 S1 次單位。本研究成功製備出經核苷酸修飾的 S1 mRNA，並利用微流體混合系統將其高效封裝於脂質奈米顆粒（LNPs）中。經由細胞試驗證實，脂質奈米顆粒的包覆能顯著提升細胞內運送效率，並於驅使細胞表達目標。更為關鍵的是，長期穩定性監測證明，此款 LNP-mRNA 疫苗配方在常規冷藏條件下能長期維持其結構完整性與轉譯能力，確保了抗原表達效能的持續與穩定。總結來說，本研究不僅首次闡明四種新型豬隻凝集素於 PEDV 感染細胞功能性角色，亦成功產製脂質奈米微粒包覆之流行性下痢病毒棘蛋白 S1 結構域信使核糖核酸疫苗平台，具高效、穩定且具備物流可行性的候選疫苗之潛力，為全球養豬業控制冠狀病毒疫情提供了創新的應對方案。

關鍵字： 豬流行性下痢病毒（PEDV）、附著因子、豬源凝集素（mKCR、Nkp44、Siglec-9、TLT2）、脂質奈米顆粒信使核糖核酸疫苗、PEDV S1 結構域

ABSTRACT



Porcine epidemic diarrhea virus (PEDV) causes severe acute watery diarrhea, acute vomiting, and high mortality in neonatal piglets, resulting in catastrophic economic losses in the global swine industry. However, controversy persists regarding its primary functional entry receptors. Given that the PEDV spike (S) protein possesses a highly glycosylated surface landscape, host glycan-lectin interactions are widely hypothesized to play a crucial role in initial viral attachment and entry into host cells. To systematically identify these host factors, this study utilized a high-throughput affinity ELISA screening to evaluate potential interactions between carbohydrate-binding lectins and the recombinant PEDV spike protein. Based on this screening, four key candidate porcine lectins, mKCR, Nkp44, Siglec-9, and TLT2, were prioritized for further evaluation. Bioinformatic analysis confirmed high sequence conservation within their carbohydrate-recognition domains (CRDs) and successfully identified their specific homologous genes within the porcine genome. To investigate the potential function of these porcine lectins in facilitating viral entry, corresponding porcine expression vectors were constructed and introduced into both naturally permissive and strictly non-permissive cellular models. Cellular and protein expression analyses verified that each candidate lectin was successfully expressed within the cytoplasm. However, in subsequent viral challenge assays, molecular detection and viral antigen staining consistently revealed a complete



absence of viral replication or viral protein synthesis in the non-permissive cellular model, regardless of candidate lectin expression. Similarly, the expression of these lectins in naturally permissive models failed to enhance or alter baseline intracellular viral loads compared to the control group. These findings collectively demonstrate that while mKCR, Nkp44, Siglec-9, and TLT2 interact with the viral glycan shield to provide physical sequestration sites on the host plasma membrane, they function strictly as non-permissive attachment factors. By anchoring virions to the cell surface through multivalent avidity, they might act as initial tethering phase of the infection cycle but are insufficient to independently trigger membrane fusion or mediate productive viral entry. Complementing these mechanistic insights into viral attachment, this study developed a messenger RNA (mRNA) vaccine strategy hoping to conquer rapid viral mutations and evolving field strains. To overcome the inherent structural complexity, cellular metabolic burden, and suboptimal protective efficacy associated with full-length spike protein formulations previously evaluated in our laboratory, this platform strategically pivoted to the S1 subunit, which concentrates the primary neutralizing epitopes and the receptor-binding domain. Nucleoside-modified S1 mRNA was generated via *in vitro* transcription and encapsulated into lipid nanoparticles (LNPs) using a specialized microfluidic mixing system. Cellular delivery assays demonstrated that LNP encapsulation significantly enhanced intracellular delivery, driving robust target protein expression within the



cytoplasm. Crucially, long-term stability monitoring proved that the formulated LNP-mRNA vaccine successfully retained its structural integrity and translational capability under standard refrigeration conditions, ensuring consistent and sustained antigen expression efficiency over time. In conclusion, this study not only provides the first functional characterization of these four novel porcine lectins during PEDV infection, but also successfully develops a lipid nanoparticle (LNP)-encapsulated PEDV S1-based mRNA vaccine platform. This platform demonstrates significant potential as a highly efficient, stable, and logistically viable vaccine candidate, offering an innovative strategy for the global swine industry to control coronaviral outbreaks.

Keywords: Porcine epidemic diarrhea virus (PEDV), attachment factor, porcine lectin (mKCR, Nkp44, Siglec-9, and TLT2), lipid-nanoparticle-mRNA vaccine, and PEDV S1 subunit

CONTENTS



中文摘要.....	i
英文摘要.....	iii
CONTENTS.....	vi
LIST OF FIGURES.....	xi
LIST OF TABLES.....	xiii
Chapter 1 General Introduction.....	1
1.1 Overview of porcine epidemic diarrhea	1
1.2 Molecular characterization of PEDV.....	3
1.2.1 Evolution and global distribution of variant strains.....	4
1.2.2 Genomic organization and virion structure.....	5
1.2.3 Glycosylation on spike protein.....	7
1.2.4 Functional role of the S1 domain.....	10
1.2.5 Host innate and adaptive immune responses.....	12
1.2.6 Beyond pAPN: evolving concepts in PEDV receptor discovery.....	13
1.2.7 Current strategies for vaccine development.....	15
1.3 Lectins: structure and pathogenic roles.....	16
1.3.1 Overview of lectins.....	16
1.3.2 Structural characteristics and biochemical properties.....	18



1.3.3	Mechanisms of lectin-viral glycan interactions.....	19
1.3.4	The functional role of lectins in viral pathogenesis.....	21
1.4	mRNA Vaccines: principles and platforms.....	22
1.4.1	Evolution and applications of mRNA technology.....	22
1.4.2	Molecular engineering and nucleoside modifications.....	24
1.4.3	Lipid nanoparticles (LNPs) as delivery vehicles.....	25
1.4.4	Physicochemical properties and endosomal escape.....	27
1.4.5	Immunological mechanisms of LNP-mRNA formulations.....	28
1.4.6	Current progress in coronaviral mRNA vaccine research.....	31
1.5	Amis of study.....	33
Chapter 2	Materials and Methods.....	34
2.1	Evaluation of the role of porcine lectins of mKCR, Nkp44, Siglec-9, and TLT2 in porcine epidemic diarrhea virus infection.....	34
2.1.1	Lectin binding assay.....	34
2.1.2	Sequence homology screening, porcine lectin genes identification, and plasmid construction.....	36
2.1.3	Plasmid transfection and expression.....	37



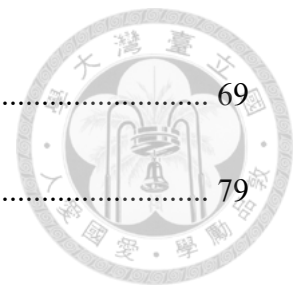
2.1.4	Western blot.....	37
2.1.5	Immunocytochemistry (ICC) staining.....	38
2.1.6	Viral propagation and cell culture.....	39
2.1.7	Viral infection and <i>in vitro</i> challenge assays.....	40
2.1.8	Viral RNA extraction and sample processing.....	41
2.1.9	Reverse transcription and real-time quantitative PCR (qPCR) analysis.....	41
2.1.10	Immunocytochemistry staining for PEDV detection.....	42
2.2	Development of lipid nanoparticle S1-based mRNA vaccine against porcine epidemic diarrhea virus.....	42
2.2.1	Plasmid preparation and construction.....	43
2.2.2	Generation of messenger RNA.....	43
2.2.3	Formulation of lipid nanoparticles (LNPs).....	45
2.2.4	Evaluation of mRNA transfection efficiency.....	46
2.2.5	Immunocytochemistry (ICC) staining.....	47
2.2.6	Western blot.....	48



2.2.7	Long-term stability assessment of LNP-encapsulated mRNA.....	49
Chapter 3	Result.....	51
3.1	Evaluation of the role of porcine lectins of mKCR, Nkp44, Siglec-9, and TLT2 in porcine epidemic diarrhea virus infection.....	51
3.1.1	High-throughput screening of PEDV spike-binding lectins.....	51
3.1.2	Generation and evaluation the expression of porcine lectins.....	51
3.1.3	Identification of MDCK as a non-permissive model for PEDV infection.....	53
3.1.4	Porcine lectins were not able to support PEDV infection in non-permissive MDCK cells quantitative analysis of viral RNA.....	55
3.2	Development of lipid nanoparticle S1-based mRNA vaccine against porcine epidemic diarrhea virus.....	58
3.2.1	Generation of lipid nanoparticles-mRNA.....	58
3.2.2	Assessment of mRNA delivery and target protein expression.....	59
3.2.3	Long-term stability and functional efficacy of LNP mRNAs.....	60
Chapter 4	Discussion.....	61

Figures.....	69
--------------	----

參考文獻.....	79
-----------	----

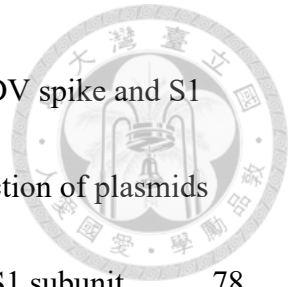


LIST OF FIGURES



Fig.1	Identification of the binding of host lectins with the PEDV spike (S) protein using the high-throughput ELISA-based screening.....	69
Fig.2	Construction and enzymatic verification of pcDNA3.1A-based porcine lectin plasmids.....	70
Fig. 3.	Validation of recombinant porcine lectin expression in HEK293 and MDCK cells.....	71
Fig. 4.	Immunocytochemical staining in detection of PEDV infection using the anti-protein antibody, PEDV inoculated in different lectin-encoded plasmids transfected MDCK cells.....	73
Fig. 5.	Linearization of the pcDNA3.1-PEDV S1-V5-His plasmid.....	74
Fig. 6.	The positive expression rate of PEDV S1 is enhanced after LNP encapsulation, as detected by ICC staining.....	75
Fig. 7.	Protein expression level of PEDV S1 detected by western blotting.....	76
Fig. 8.	The stability of LNP-encapsulated PEDV S1 mRNA over a three-month storage period.....	77

Fig. 9. Comparison of protein expression levels of full-length PEDV spike and S1 subunit in HEK293 cell lysates following transient transfection of plasmids encoding either the full-length PEDV spike protein or the S1 subunit..... 78



LIST OF TABLES



Table 1. Identification and annotation of porcine lectin homologs.....	53
Table 2. The permissibility of cell lines to PEDV Infection.....	54
Table 3. Real-time qPCR quantification of average viral load (log/mL) in candidate lectin-expressing MDCK cells.....	57
Table 4. Real-time qPCR quantification of viral RNA in cell lysate of transfected HEK293 cells at 48hpi.....	58
Table 5. The LNP-mRNA encapsulation and recovery rate.....	59



Chapter 1. Introduction

1.1 Overview of porcine epidemic diarrhea

Porcine epidemic diarrhea virus (PEDV) has been recognized as one of the most devastating enteric pathogens in the global swine industry for over half a century. The virus was first identified during a series of severe diarrheal outbreaks on swine breeding farms in the United Kingdom (1971) and subsequently in Belgium (1978) (Pensaert & De Bouck, 1978; Wood, 1977). While early European outbreaks, which were associated with the prototype CV777 strain, were relatively sporadic, the epidemiological landscape shifted dramatically in the 21st century. The emergence of highly virulent non-S INDEL (Genogroup 2) strains in China in 2010, and the subsequent catastrophic spread to the United States in 2013, caused the loss of over 10% of the annual US pig crop within a single year (Huang et al., 2013; Jung & Saif, 2015; Lin et al., 2016). Nowadays, PEDV remains an endemic threat across Asia, Europe, and the America, resulting in an expensive, repeating cycle with no end in sight.

As a highly contagious alphacoronavirus, PEDV manifests clinically as severe acute watery diarrhea, vomiting, life-threatening dehydration, and even high mortality. Although the virus is capable of infecting swine of all ages, its impact is particularly catastrophic in neonatal piglets. In naive herds, mortality rates for piglets under one week

of age frequently approach 100%. This high lethality is a direct consequence of the slow regenerative capacity of the neonatal porcine intestine. Once the virus destroys the absorptive surface, the resulting electrolyte imbalance and metabolic acidosis lead to rapid clinical decline and death (Jung et al., 2020; Li et al., 2016).

The primary tropism of PEDV is directed toward the mature villous enterocytes of the jejunum and ileum. Upon infection, these target cells undergo significant necrosis and syncytia formation, leading to severe structural damage. A hallmark of PEDV pathology is the dramatic reduction in the villous height to crypt depth (VH:CD) ratio (Stevenson et al., 2013). This morphological destruction results in severe osmotic malabsorption, as the damaged intestine can no longer process essential nutrients from sow's milk. While older pigs exhibit an age-dependent resistance due to more rapid enterocyte turnover and a more mature immune system, they still serve as significant viral reservoirs that contribute to the persistence of the virus within a facility (Jung & Saif, 2015; Jung et al., 2020).

Despite intensive research and the implementation of various vaccination protocols, PEDV outbreaks remain widely difficult to control. Several factors contribute to this lack of effective containment, including the high environmental stability and low infectious dose (Jung & Saif, 2015; Jung et al., 2020), rapid viral evolution (Lin et al., 2016), and the limitations of mucosal immunity (Langel et al., 2016). PEDV is highly stable in cool,

moist environments and can be disseminated rapidly via contaminated transport vehicles, personnel, and even feed (Jung & Saif, 2015; Stevenson et al., 2013). A remarkably low dose of viral particles is sufficient to initiate an outbreak in a susceptible population.

Moreover, the PEDV genome exhibits high rates of mutation and recombination, particularly within the spike (S) gene (Lin et al., 2016). This genetic plasticity allows the virus to evade existing maternal immunity and contributes to vaccine failure when using older, strain-specific products (Lin et al., 2016; Wang et al., 2016). Even more concerning, protecting neonatal piglets requires the induction of robust secretory IgA (sIgA) in the sow's colostrum and milk, a process known as the lactogenic immune axis (Jung & Saif, 2015; Langel et al., 2016). Traditional inactivated or injectable vaccines often fail to induce sufficient mucosal immunity in the gut, leaving piglets vulnerable during the most critical first week of life (Jung et al., 2020).

Given these persistent challenges, there is an urgent need to redefine the molecular mechanisms of PEDV attachment and to develop next-generation vaccine platforms, such as LNP-mRNA technologies (Chaudhary et al., 2021; Pardi et al., 2018b), that can provide more precise and potent protection against evolving viral strains (Jung et al., 2020; Lin et al., 2016).

1.2 Molecular characterization of PEDV

1.2.1 Evolution and global distribution of variant strains



Since its initial discovery, PEDV has undergone significant genetic evolution, leading to the emergence of diverse strains with varying levels of virulence. Phylogenetically, global PEDV isolates are categorized into two primary genogroups: Group 1 (G1) and Group 2 (G2), which are further divided into sublineages (1a, 1b, 2a, 2b, and 2c) based on the genetic diversity of the S protein (Lin et al., 2016; Wang et al., 2016).

The G1 strains represent the classical variants, including the prototype Belgian CV777 strain and early vaccine strains used in Asia. For decades, these strains caused relatively mild or sporadic outbreaks. However, a major evolutionary shift occurred around 2010 in China with the emergence of G2 strains, often referred to as non-S INDEL variants. These highly virulent strains are characterized by specific insertions and deletions (INDELs) and multiple point mutations within the S1 domain of the spike protein, which is the primary target for neutralizing antibodies (Jung & Saif, 2015; Lin et al., 2016).

In 2013, these G2 strains were catastrophically introduced into the United States, likely via contaminated bulk feed ingredients, and subsequently spread to Canada and Mexico. This North American outbreak resulted in a massive loss of neonatal piglets and

confirmed the transcontinental transmission capability of the virus (Stevenson et al., 2013). In Taiwan and Southeast Asia, the epidemiological landscape is dominated by G2b variants, which have shown persistent circulation and an ability to evade maternal immunity induced by traditional G1-based inactivated vaccines (Lin et al., 2016).

The continuous evolution of PEDV is driven by the high error rate of the viral RNA-dependent RNA polymerase (RdRp) and frequent recombination events within the S gene. This genetic plasticity results in significant antigenic drift, complicating control efforts. Recent studies highlight that G2 strains possess a distinct glycan-binding profile compared to G1 strains, potentially altering their cell tropism and interaction with host lectins (Cui et al., 2020; Jung et al., 2020). Consequently, understanding the global distribution and evolutionary trajectory of these variant strains is critical for the rational design of next-generation LNP-mRNA vaccines that can provide broad protection against current circulating field isolates.

1.2.2 Genomic organization and virion structure

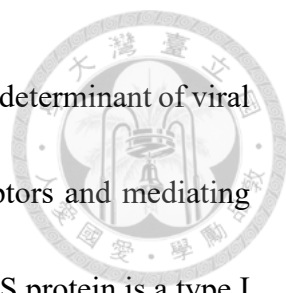
Porcine epidemic diarrhea virus is a taxonomically classified member of the genus Alphacoronavirus within the family *Coronaviridae*. The virion is characterized by an enveloped, pleomorphic structure with a diameter ranging from 95 to 190 nm (Pensaert & De Bouck, 1978). Its genetic blueprint consists of a single-stranded, positive-sense

RNA genome of roughly 28 kb, which is capped at the 5' end and polyadenylated at the 3' end.



The PEDV genome follows a characteristic coronaviral organization: 5'-UTR-ORF1a/1b-S-ORF3-E-M-N-3'-UTR. The large 5' proximal open reading frames, ORF1a and ORF1b, occupy nearly two-thirds of the genome and encode two massive polyproteins (pp1a and pp1ab). These are proteolytically processed by viral proteases into sixteen non-structural proteins (nsp1-nsp16), which assemble into the replicase-transcriptase complex (RTC) responsible for viral RNA synthesis and proofreading. The remaining third of the genome encodes the accessory protein ORF3, a putative ion channel, and the four essential structural proteins, including the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (Kocherhans et al., 2001; Wang et al., 2012).

The virion structure is maintained by these structural components. The N protein encapsulates the viral RNA to form the helical ribonucleocapsid, while the M protein, the most abundant envelope component, dictates the shape of the virion and coordinates assembly through interactions with other structural proteins (Jung & Saif, 2015). The E protein is a small viroporin essential for viral morphogenesis and assembly (Zhang et al., 2022).

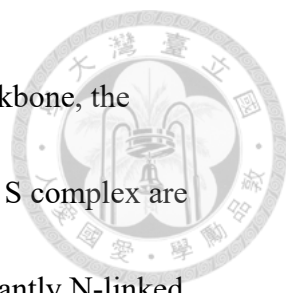


Among these structural components, the S protein is the primary determinant of viral pathogenesis (Lin et al., 2016), facilitating attachment to host receptors and mediating membrane fusion (Cui et al., 2020; Li. et al., 2016). Structurally, the S protein is a type I transmembrane glycoprotein that assembles into homo-trimers on the viral surface, giving the virus its crown-like appearance. It is functionally divided into two subunits: the N-terminal S1 subunit (aa 1-726), which contains the receptor-binding domain (RBD) for initial host cell recognition, and the C-terminal S2 subunit (aa 727-1386), which anchors the protein to the viral envelope and contains the machinery for membrane fusion (Li. et al., 2016)

Notably, the S protein is heavily glycosylated, presenting a dense glycan shield composed of N-linked oligosaccharides. This carbohydrate coat serves a dual purpose: it shields critical neutralizing epitopes from the host immune system and acts as a complex interface for interacting with various host-cell surface molecules and lectins, such as sialic acids and DC-SIGN (Zhao et al., 2021), extending the viral tropism beyond traditional proteinaceous receptors (Li. et al., 2016; Lin et al., 2016)

1.2.3 Glycosylation on spike protein

The entry and structural integrity of PEDV are governed by its surface-exposed S glycoprotein, a heavily glycosylated homo-trimeric Class I fusion machinery (Tortorici



& Veessler, 2019). Beyond the primary proteinaceous amino acid backbone, the topographical architecture and biophysical dynamic properties of the S complex are extensively remodeled by post-translational modifications, predominantly N-linked glycosylation (Huang et al., 2022). High-resolution cryo-electron microscopy (cryo-EM) and site-specific mass spectrometry analysis indicate that each S monomer possesses a high density of conserved asparagine-X-serine/threonine (Asn-X-Ser/Thr) sequons, which are densely cloaked by host-cell-derived oligosaccharides, collectively functioning as a sophisticated dynamic glycan shield (Huang et al., 2022; Watanabe et al., 2020).

Within the structural framework of the peripheral S1 subunit, the primary domain responsible for initial host interaction, this glycan shield is highly heterogeneous, comprising three fundamental biosynthetic carbohydrate classifications: high-mannose-type, hybrid-type, and fully processed complex-type glycans (Tsai et al., 2024; Watanabe et al., 2020). High-mannose glycans (Man₅-Man₉) are typically sequestered within the sterically restricted trimeric interfaces and inner structural cavities where processing enzymes lack spatial accessibility (Watanabe et al., 2020). Conversely, the solvent-exposed outer canopy of the S1 domain, including the sialic acid-binding Domain 0 (D0), the adjacent N-terminal domain (NTD), and the C-terminal receptor-binding domain (CTD), is heavily enriched with highly branched, fully mature

complex-type glycans adorned with terminal galactose, fucose, and highly negatively charged terminal sialic acids (Neu5Ac) (Huang et al., 2022; Van Breedam et al., 2014).



From an immunological perspective, this intricate glycan canopy operates as a double-edged sword in coronavirus pathogenesis. Primarily, the dynamic spatial movement of these complex-type glycans projects severe steric hindrance over the rigid proteinaceous core, effectively camouflaging highly conserved neutralizing epitopes from the host's humoral immune surveillance and B-cell receptor recognition (Tortorici & Veasler, 2019). However, structural vaccine investigations targeting Domain A-specific neutralizing epitopes demonstrate that preserving the native, mammalian-cell-derived glycosylation profile is crucial for maintaining accurate antigenicity (Chang et al., 2021). Indeed, as demonstrated by Huang et al. (2022), these biologically authentic glycan conformations are structurally indispensable for stabilizing the flexible domains and maintaining the accurate topological folding of key neutralizing targets.

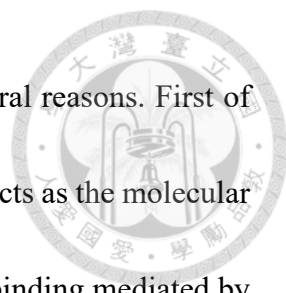
Furthermore, computational simulations using advanced glycan conformer libraries corroborate that these host-derived carbohydrates are not mere passive shields. Their local steric constraints and spatial exclusions actively lock the trimeric S protein in functional orientations, thereby fine-tuning the thermodynamic equilibrium between the up (receptor-accessible) and down (immune-evasive) conformational states (Tsai et al., 2024).

1.2.4 Functional role of the S1 subunit



The S protein of PEDV is a massive, homotrimeric class I transmembrane glycoprotein that coordinates the complex process of viral entry. Each monomeric S protein is synthesized as a precursor that is functionally demarcated into two distinct regions: the N-terminal S1 subunit and the C-terminal S2 subunit (Li. et al., 2016). While the S2 subunit is highly conserved and contains the essential machinery for membrane fusion, including the fusion peptide (FP) and heptad repeat regions (HR1 and HR2), the S1 subunit is characterized by high genetic plasticity and serves as the primary interface between the virus and the host cell (Lin et al., 2016; Song & Park, 2012).

The S1 subunit (aa 1-726) is the principal determinant of viral tropism and host range. Structural and biochemical characterizations have revealed that the PEDV S1 subunit possesses a modular architectural organization, conventionally sub-functionalized into distinct structural domains: Domain 0, the N-terminal domain (also referred to as Domain A), the C-terminal domain (also referred to as Domain B), and the subsequent subdomains (SD1/SD2, often designated as Domain C/D) (Chang et al., 2021; Huang et al., 2022). These subdomains could function cooperatively as RBDs or attachment interfaces that orchestrate the multi-step viral docking cascade onto host enterocytes.



The S1 subunit plays a crucial role in PEDV infection for several reasons. First of all, it provides a dock for initial attachment of virus. The S1 subunit acts as the molecular key that senses and captures the host cell. Without the high-affinity binding mediated by S1 subunit, the virus cannot stabilize itself on the apical membrane of enterocytes to initiate the subsequent fusion process. Additionally, the S1 subunit exhibits clear antigenic dominance, eliciting a robust neutralizing antibody response. It contains the majority of the neutralizing epitopes (such as the COE domain, aa 499-638). Consequently, it is the primary target for the host's humoral immune response. Antibodies that bind to S1 can sterically hinder viral attachment, effectively neutralizing the virus before it can deliver its genome into the cytoplasm (Chang et al., 2002; Wang et al., 2016). Furthermore, mutations in the S1 subunit provide viral adaptation to different host cell receptors. Mutations within the S1 subunit, particularly in the RBD regions, allow PEDV to adapt to different host environments or evade existing maternal immunity. This explains why S1 is the most variable region in the genome and why it is the focal point for monitoring the evolution of G1 and G2 strains (Lin et al., 2016).

Given its indispensable role in receptor recognition and its status as a potent immunogen, the S1 subunit represents the most logical candidate for the development of targeted prophylactic strategies. This emphasizes the reason for utilizing an LNP S1-

based mRNA vaccine platform, which aims to induce a robust and precise immune response against this critical entry mediator.



1.2.5 Host innate and adaptive immune responses

The host immune response to PEDV involves a complex interplay between innate viral recognition and the subsequent activation of adaptive immunity, both of which are significantly hindered in neonatal piglets. Upon infection, PEDV is recognized by pattern recognition receptors (PRRs), such as TLR3 and RIG-I, which normally trigger a protective Type I and III interferon (IFN) response. However, PEDV has evolved various non-structural proteins (e.g., nsP1, nsP3, nsP15, and nsP16) and the nucleocapsid (N) protein to actively antagonize these pathways, effectively suppressing the host's innate antiviral alarm (Deng et al., 2019; Zhang et al., 2018). This evasion is particularly devastating for neonatal piglets, whose innate immune sensors are physiologically less responsive than those of adult swine (Annamalai et al., 2015; Jung et al., 2020). Furthermore, the adaptive immune response in piglets is characterized by a window of vulnerability. Because of the epitheliochorial placenta in swine, maternal antibodies cannot be transferred in utero, leaving piglets born agammaglobulinemic and entirely dependent on the lactogenic immune axis (Langel et al., 2016). While older pigs can mount an endogenous sIgA response in the gut-associated lymphoid tissue (GALT),

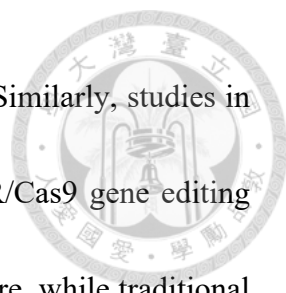
neonates rely solely on the continuous supply of sIgA from the sow's colostrum and milk to neutralize the virus within the intestinal lumen (Jung & Saif, 2015; Jung et al., 2020).

The high mortality observed in neonatal herds is often a direct result of this perfect storm: a highly evasive viral mechanism coupled with a naive neonatal immune system and insufficient levels of passive sIgA transfer from the mother (Langel et al., 2016).

1.2.6 Beyond pAPN: Evolving concepts in PEDV receptor discovery

For over two decades, porcine aminopeptidase N (pAPN), a 150-kDa metallotranspeptidase expressed on the brush border of intestinal epithelia, has been identified as the primary functional receptor for PEDV (Li. et al., 2016). Early studies demonstrated that heterologous expression of pAPN in non-permissive cell lines made them susceptible to infection, reinforcing the consensus that pAPN serves as the critical gateway for the virus (Li et al., 2007; Nam & Lee, 2010).

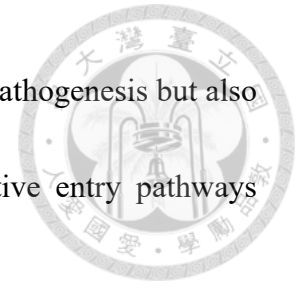
However, recent evidence utilizing CRISPR/Cas9 technology has introduced significant controversy regarding the absolute indispensability of pAPN for viral entry. Advanced gene knockout studies have demonstrated that while the loss of pAPN did not significantly affect viral replication, proving it is not the essential receptor for entry and replication. Specifically, in ST cells where the ANPEP gene was successfully disrupted using CRISPR/Cas9, PEDV remained capable of infecting the cells and initiating



replication at levels comparable to wild-type cells (Li et al., 2017). Similarly, studies in Vero cells, a classic permissive model, confirmed through CRISPR/Cas9 gene editing that pAPN is not the sole restrictive factor for viral entry. Furthermore, while traditional gene silencing approaches, such as siRNA-mediated knockdown in IPEC-J2 cells (Ji et al., 2018), have consistently shown reduced viral susceptibility, the persistence of replication in these models reinforces the notion that pAPN may function more as a sequestration factor or attachment scaffold. These findings collectively suggest that the definitive entry process likely requires the synergistic involvement of alternative co-factors or secondary entry mediators beyond the canonical pAPN receptor (Cui et al., 2020; Ji et al., 2018; Li et al., 2017).

These findings collectively suggest that the definitive entry process likely requires the synergistic involvement of alternative co-factors or secondary entry mediators (Ji et al., 2018; Li et al., 2017). Beyond proteinaceous receptors, emerging evidence highlights the role of sialic acids and host lectins as critical attachment factors (Zhao et al., 2021). The S1-NTD of the PEDV spike protein has been shown to possess carbohydrate-binding activity, facilitating initial docking on the glycan-rich surface of the intestinal epithelium (Deng et al., 2016; Li. et al., 2016). This shifting landscape suggests that PEDV utilizes a multi-step entry mechanism, where various co-factors modulate viral tropism and enhance the efficiency of membrane fusion (Li. et al., 2016). Consequently, identifying

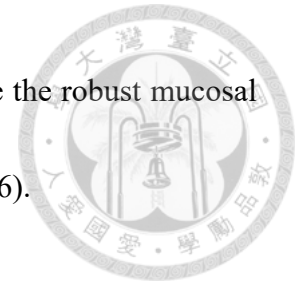
these novel interactors is essential not only for understanding viral pathogenesis but also for the strategic design of vaccines that can block these alternative entry pathways effectively.



1.2.7 Current strategies for vaccine development

Since the discovery of PEDV, vaccine strategies have evolved from classical whole-virus platforms to advanced recombinant technologies, yet effective control remains subtle. Historically, live-attenuated vaccines (LAVs) and inactivated vaccines based on the G1-genogroup (e.g., CV777 and DR13 strains) were widely utilized in Asia; however, these showed poor cross-protection against the highly virulent G2 variant strains that emerged post-2010 (Lin et al., 2016; Song & Park, 2012). A critical factor further complicating vaccine development is the uncertainty surrounding the conclusive primary receptor for PEDV. The recent shift away from the pAPN-centric model has created a significant bottleneck in identifying the precise neutralizing epitopes required for sterile immunity (Li et al., 2017). Without a definitively identified cellular receptor acting as the molecular lock, designing structure-based vaccines capable of precisely obstructing the viral keys remains a formidable challenge. In response to global outbreaks, several G2-based vaccines were conditionally licensed in the United States and other Asian countries, including inactivated whole-virus and viral-vector platforms (Gerdtz & Zakhartchouk,

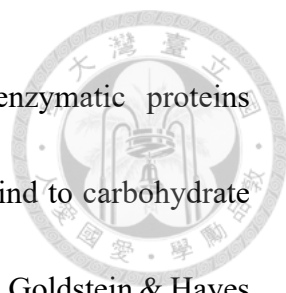
2017). Despite these efforts, traditional vaccines often fail to induce the robust mucosal sIgA necessary for effective lactogenic immunity (Langel et al., 2016).



In Taiwan, the epidemiological landscape is dominated by G2b variants, yet there are currently no commercially licensed PEDV vaccines available for use (Chiou et al., 2017; Lin et al., 2014). Consequently, many Taiwanese producers rely on feedback protocols, which involves in the controlled oral exposure of sows to infected piglet materials to stimulate maternal immunity (Lin et al., 2016). While feedback can induce sIgA, it poses severe biosafety risks, including the potential for spreading co-pathogens and maintaining environmental viral reservoirs (Jung et al., 2020; Song & Park, 2012). This significant gap in standardized tools, compounded by the complexity of viral entry mechanisms involving alternative co-factors (Zhao et al., 2021), underscores the urgent need for next-generation platforms. LNP-mRNA vaccines offer a promising solution, providing rapid sequence adaptability to match evolving variants and a superior safety profile to overcome the limitations of traditional methods (Chaudhary et al., 2021; Pardi et al., 2018b).

1.3 Lectins: structure and pathogenic roles

1.3.1 Overview of lectins



Lectins represent a diverse family of non-antibody, non-enzymatic proteins characterized by their unique ability to specifically and reversibly bind to carbohydrate moieties without altering their covalent structures (Gabius et al., 2002; Goldstein & Hayes, 1978). Historically, while these proteins were initially recognized for their potent hemagglutinating properties in plant extracts, the field underwent a transformative shift in 1954 when William C. Boyd coined the term lectin, derived from the Latin *legere*, meaning to pick or select, to reflect their remarkable capacity to distinguish between specific blood group antigens and complex sugar structures (Boyd & Shapleigh, 1954). Biologically, the function of lectins is dictated by their conserved carbohydrate-recognition domains (CRDs), which allow them to decode the sugar code expressed on the surfaces of glycoproteins and glycolipids (Gabius et al., 2002; Weis & Drickamer, 1996). This molecular specificity enables lectins to mediate a wide array of critical physiological processes, including cell-cell adhesion, intracellular protein trafficking, and innate immune surveillance. Within the context of host-pathogen interactions, many lectins, particularly C-type lectins that utilize ions for binding, play a dual role. Although they function as pattern recognition receptors for the host immune system, they are frequently hijacked by enveloped viruses as attachment factors to facilitate initial docking on the host cell membrane (Van Breedam et al., 2014). Consequently, the structural and

functional diversity of lectins remains fundamental to understanding the complex glycan-mediated interactions that govern viral entry and subsequent pathogenesis.



1.3.2 Structural characteristics and biochemical properties

The structural and biochemical diversity of lectins is fundamental to their capacity to decode complex glycan signatures. Structurally, lectins are defined by their highly conserved CRDs, which vary significantly in their fold architecture depending on the lectin family. For instance, C-type lectins (CTLs) utilize a characteristic fold consisting of two α -helices and two anti-parallel β -sheets (Zelensky & Gready, 2005), whereas Galectins typically exhibit a jelly-roll-sandwich fold (Gabius et al., 2002). A critical structural hallmark of many lectins is their multivalent nature; many exist as dimers, trimers, or higher-order oligomers. This multivalent presentation significantly enhances their functional avidity, allowing for high-affinity binding to clustered glycan ligands on the cell surface, a phenomenon often referred to as the velcro effect (Dam & Brewer, 2010; Weis & Drickamer, 1996).

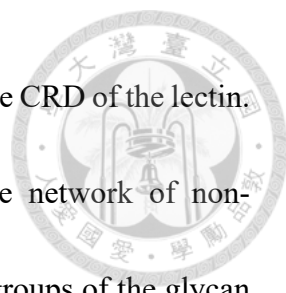
Biochemically, lectins are characterized by their specificity for particular monosaccharides or oligosaccharide motifs, such as mannose, galactose, or acetylglucosamine. This specificity is achieved through a network of hydrogen bonds, van der Waals forces, and hydrophobic interactions, such as packing of a hydrophobic



sugar face against aromatic amino acid side chains, within the binding pocket (Weis & Drickamer, 1996). A significant subset of lectins are metallo-proteins that require divalent cations for their function, albeit through distinct mechanisms. For instance, legume lectins utilize and primarily to stabilize the CRD architecture, whereas the CTL family uniquely requires to form direct coordination bonds with the hydroxyl groups of the sugar ligand (Weis & Drickamer, 1996; Zelensky & Gready, 2005). Lectins bind to their targets reversibly and non-covalently without altering the carbohydrate's structure, making them robust biochemical tools for studying cellular glycosylation patterns and host-pathogen interactions (Gabius et al., 2002; Goldstein & Hayes, 1978; Sharon & Lis, 2004).

1.3.3 Mechanisms of lectin-viral glycan interactions

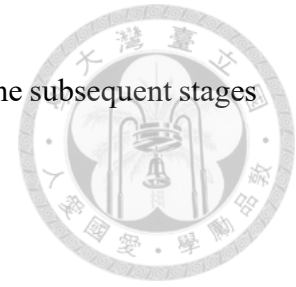
The initiation of viral infection is a complicated molecular recognition event dominated by the biochemical interplay between host lectins and viral glycans. Enveloped viruses, particularly coronaviruses like PEDV, utilize a dense glycan shield on their S proteins to navigate the host environment. This shield is composed of a diverse array of oligosaccharides, ranging from high-mannose-type glycans to complex-type structures capped with sialic acids (Watanabe et al., 2020). These glycans serve as the primary ligands for host lectins, which act as biological sensors that read the specific sugar patterns displayed on the viral surface (Gabius et al., 2002).



The physical chemistry of these interactions is centered within the CRD of the lectin. Molecular docking and specificity are achieved through a precise network of non-covalent forces, including hydrogen bonding between the hydroxyl groups of the glycan and the amino acid side chains of the lectin, often supplemented by van der Waals forces and hydrophobic stacking (Weis & Drickamer, 1996). For C-type lectins, this interaction is strictly dependent on environmental factors, specifically the presence of divalent cations like Ca^{2+} . The calcium ion resides at the base of the binding pocket, directly coordinating with the 3- and 4-hydroxyl groups of the sugar moiety. This coordination acts as a molecular bridge, orienting the glycan in a specific conformation that maximizes binding stability (Drickamer, 1992; Zelensky & Gready, 2005).

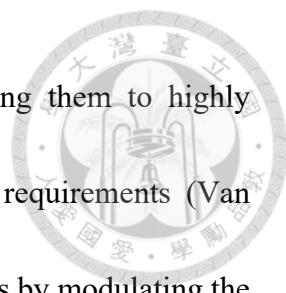
Despite the high specificity of the CRD, the affinity of a single binding event is typically weak, with dissociation constants (K_d) often in the millimolar range (Weis & Drickamer, 1996). To compensate for this, lectins utilize multivalency to achieve functional avidity. Most lectins exist as oligomers, such as dimers, trimers, or tetramers, allowing them to engage multiple glycans simultaneously on the viral S protein. This spatial clustering creates a velcro effect, where the collective strength of multiple weak interactions provides the high-affinity tethering required to capture the virus under physiological conditions (Dam & Brewer, 2010). This multivalent engagement is not merely a binding enhancer but a fundamental requirement for the virus to overcome the

low intrinsic affinity of individual sugar-binding events and initiate the subsequent stages of the infection cycle.



1.3.4 The functional role of lectins in viral pathogenesis

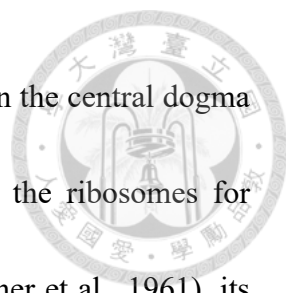
In the complex landscape of viral pathogenesis, lectins function as a double-edged sword, serving both as critical components of innate immune surveillance and as hijacked facilitators for viral entry and dissemination. While soluble host lectins, such as mannose-binding lectin (MBL) and surfactant proteins, act as a primary defense by aggregating virions or triggering complement-mediated lysis, many enveloped viruses have evolved strategies to exploit membrane-associated lectins to overcome host barriers (Van Breedam et al., 2014). This interplay significantly influences viral tissue tropism, where the specific expression patterns of host lectins across anatomical sites act as a molecular zip code that dictates viral localization. For enteric pathogens like PEDV, the dense expression of specific lectins within the porcine intestinal mucosa likely serves as a critical determinant for localized infection by concentrating viral particles or capturing them via subepithelial immune cells (Zhao et al., 2021). Beyond simple attachment, lectins play a pivotal role in mediating viral entry through diverse pathways. For instance, C-type lectins like DC-SIGN (CD209) and L-SIGN (CD209L) can directly mediate *cis*-infection in otherwise refractory cells (Zhao et al., 2021), or drive the specialized process



of *trans*-infection by capturing virions and subsequently presenting them to highly susceptible target cells, effectively bypassing traditional receptor requirements (Van Breedam et al., 2014). Furthermore, lectins contribute to pathogenesis by modulating the host's immune landscape; for instance, the interaction between the viral S protein and inhibitory receptors such as the siglec family can suppress innate immune signaling, inhibiting pro-inflammatory cytokine production to create a permissive environment for replication (Crocker et al., 2007). Conversely, aberrant lectin-viral engagement can trigger the over-activation of intracellular signaling pathways, potentially contributing to the severe mucosal inflammation or cytokine storms observed in acute infections (Osorio & e Sousa, 2011). The persistence of PEDV infection in pAPN-deficient environments highlights a significant gap in our understanding, necessitating the characterization of specific host lectins such as mKCR (CLEC4F), Nkp44 (NCR2), Siglec-9 (CD33-like), and TLT2 (TREM2) to validate a synergistic entry model that accounts for stabilized viral docking, immune evasion, and tissue-specific dissemination within the global swine industry.

1.4 mRNA vaccines: principles and platforms

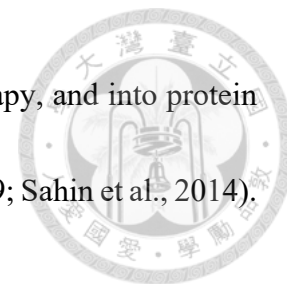
1.4.1 Evolution and applications of mRNA technology



Messenger RNA (mRNA) serves as the transient intermediary in the central dogma of molecular biology, carrying genetic instructions from DNA to the ribosomes for protein synthesis. While mRNA was first discovered in 1961 (Brenner et al., 1961), its development into a therapeutic platform was historically hindered by its inherent instability and the potent inflammatory responses it triggered in host cells (Pardi et al., 2018b). The field underwent a transformative evolution with the advent of *in vitro* transcribed (IVT) mRNA and the landmark discovery that incorporating modified nucleosides, such as pseudouridine, significantly reduces the activation of innate immune receptors like TLR3 and TLR7, thereby enhancing both safety and protein translation efficiency (Karikó et al., 2005; Pardi et al., 2018b).

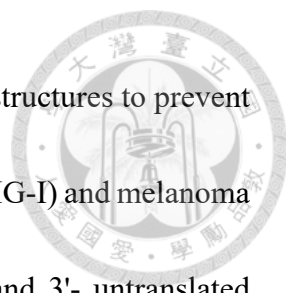
The functional success of mRNA technology is fundamentally tied to the development of complicated delivery systems, particularly lipid nanoparticles (LNPs). These ionizable lipid-based carriers protect the synthetic mRNA from enzymatic degradation and facilitate its cytoplasmic entry via endocytosis, a critical requirement for effective antigen expression (Kowalski et al., 2019; Pardi et al., 2018b). The global response to the COVID-19 pandemic served as a definitive evidence, demonstrating that mRNA vaccines can be designed, manufactured, and deployed with unprecedented speed compared to traditional platforms (Chaudhary et al., 2021). Beyond infectious diseases, the applications of mRNA technology are rapidly expanding into oncology, where it is

used to encode personalized tumor antigens for cancer immunotherapy, and into protein replacement therapies for rare genetic disorders (Kowalski et al., 2019; Sahin et al., 2014).



1.4.2 Molecular engineering and nucleoside modifications

The clinical viability of mRNA as a therapeutic platform is the result of decades of complicated molecular engineering designed to overcome the molecule's inherent limitations, such as the high immunogenicity and rapid enzymatic degradation. The most transformative breakthrough in this field was the discovery of nucleoside modifications, which fundamentally altered the host's innate immune perception of synthetic mRNA. Naturally occurring mRNA contains various modified nucleosides that distinguish self from non-self RNA. However, early *in vitro* transcribed (IVT) mRNA lacked these signatures, leading to the potent activation of Toll-like receptors (TLRs), particularly TLR3, TLR7, and TLR8 (Karikó et al., 2005). Landmark research demonstrated that the incorporation of modified nucleosides, most notably pseudouridine or its derivative 1-methylpseudouridine, effectively allows the mRNA to evade detection by these pattern recognition receptors, suppressing inflammatory cytokine production and significantly enhancing translational capacity by preventing the activation of protein kinase R (Karikó et al., 2008; Pardi et al., 2018b). Beyond nucleoside chemistry, the molecular engineering of the mRNA transcript involves the optimization of several structural elements to



maximize stability and protein yield, such as the transition to Cap-1 structures to prevent recognition by cytosolic sensors like retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), the engineering of 5'- and 3'- untranslated regions (UTRs) to extend the transcript's cytoplasmic half-life, and the strategic configuration of the poly-A tail to ensure efficient recruitment of the translation initiation complex (Chaudhary et al., 2021; Sahin et al., 2014). Furthermore, synonymous codon optimization is frequently employed to increase GC content and avoid rare tRNAs, thereby accelerating the elongation rate of the polypeptide chain (Pardi et al., 2018b). Together, these molecular refinements have transitioned mRNA from a fragile laboratory tool into a robust pharmacological agent, ensuring that synthetic instructions, such as those encoding the PEDV S1 subunit, persist long enough to induce a potent and sustained immune response within the host.

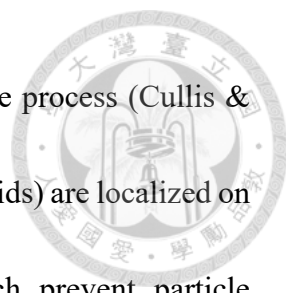
1.4.3 Lipid nanoparticles (LNPs) as delivery vehicles

LNPs have emerged as the primary delivery platform for nucleic acid therapeutics, specifically mRNA, due to their unparalleled ability to encapsulate and protect fragile genetic cargo from ubiquitous ribonucleases (RNases) and enzymatic degradation (Pardi et al., 2018b; Sahin et al., 2014). Unlike traditional viral vectors, LNPs offer a non-viral alternative characterized by low immunogenicity, high modularity, and the capacity for



scalable manufacturing, which was most notably demonstrated by the rapid global deployment of mRNA-based COVID-19 vaccines (Chaudhary et al., 2021; Schoenmaker et al., 2021). The most critical advantage of the LNP system lies in its ability to facilitate efficient cellular uptake and subsequent endosomal escape, ensuring that the encapsulated mRNA reaches the cytoplasm, the site of translation, without being degraded in the lysosomal pathway (Hou et al., 2021; Kowalski et al., 2019).

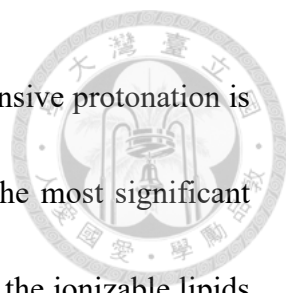
The functional success of an LNP is dictated by its complex multicomponent architecture, typically comprising four distinct lipid species, including ionizable lipids, cholesterol, helper phospholipids, and PEGylated lipids, each serving a specific role in the particle's stability and bioactivity (Cullis & Hope, 2017). Ionizable lipids are considered the functional core of the LNP; they remain neutral at physiological pH (pH 7.4) to minimize systemic toxicity but become protonated and positively charged within the acidic environment of the endosome (pH < 6.0), a transition that promotes membrane fusion and the release of the mRNA into the cytosol (Schoenmaker et al., 2021). Cholesterol is incorporated as a structural stabilizer, filling gaps within the lipid bilayer to enhance the rigidity and integrity of the nanoparticle while also influencing its fluidity and uptake efficiency (Hou et al., 2021). Helper lipids, such as distearoylphosphatidylcholine (DSPC) or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), provide a stable structural framework and assist in the



formation of the lipid shell, further supporting the endosomal escape process (Cullis & Hope, 2017). Finally, PEG-lipids (polyethylene glycol-conjugated lipids) are localized on the particle surface to provide immune-evasive properties, which prevent particle aggregation during synthesis, control the final particle size, and extend circulation half-life by reducing opsonization and clearance by the mononuclear phagocyte system (Hou et al., 2021; Pardi et al., 2018b). Together, these molecular components create a highly efficient vehicle capable of delivering complex genetic instructions to host cells for sustained protein expression.

1.4.4 Physicochemical properties and endosomal escape

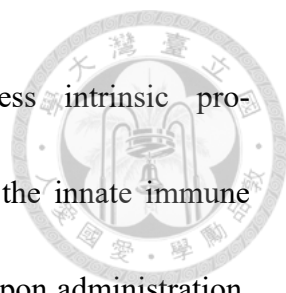
The therapeutic efficiency of LNPs is fundamentally dictated by their physicochemical properties, most notably particle size, polydispersity index (PDI), encapsulation efficiency (EE), and the acid dissociation constant (pKa) of the ionizable lipid. While a uniform particle size (typically 60–100 nm) and a low PDI (< 0.2) are essential for consistent cellular uptake via clathrin-mediated endocytosis, the of the ionizable lipid serves as the primary master switch for biological activity. An optimal pKa, typically ranging between 6.0 and 6.7, ensures that the LNP remains near-neutral at physiological pH (pH7.4) to minimize systemic toxicity and premature clearance, yet undergoes a rapid charge transition in the acidic environment of the maturing endosome



(pH 5.5-6.5) (Cullis & Hope, 2017; Hou et al., 2021). This pH-responsive protonation is the key driver of endosomal escape, which is widely regarded as the most significant efficiency bottleneck in mRNA delivery. As the endosome acidifies, the ionizable lipids acquire a positive charge and engage in potent electrostatic interactions with anionic phospholipids, such as bis(monoacylglycero)phosphate (BMP), locating in the endosomal membrane. These interactions promote a structural transition from a stable lamellar bilayer to a fusogenic inverted hexagonal phase (H_{II}), which effectively destabilizes the endosomal membrane architecture (Cullis & Hope, 2017; Schoenmaker et al., 2021). This targeted disruption facilitates the release of the encapsulated mRNA into the cytosol, where it becomes accessible to the ribosomal machinery for protein synthesis (Pardi et al., 2018b; Schoenmaker et al., 2021). Enhancing the efficiency of this escape process is essential for maximizing the potency of mRNA-based therapeutics as only a small fraction (often estimated at less than 2%) of internalized mRNA typically survives the endocytic pathway to achieve functional translation (Kowalski et al., 2019).

1.4.5 Immunological mechanisms of LNP-mRNA formulations

The immunological efficacy of LNP-mRNA formulations arises from a synergistic interplay between the synthetic mRNA cargo and the lipid delivery vehicle, which together function as a self-adjuvanted vaccine system. Beyond their role in protecting and



delivering genetic material, lipid nanoparticles (LNPs) possess intrinsic pro-inflammatory properties that act as a potent adjuvant, stimulating the innate immune system to create a milieu favorable for robust adaptive responses. Upon administration, LNPs trigger the recruitment and activation of various innate immune cells, particularly dendritic cells (DCs) and macrophages, through the induction of specific cytokines and chemokines at the injection site (Alameh et al., 2021; Pardi et al., 2018b). This adjuvant effect is crucial for the maturation of DCs, which subsequently migrate to draining lymph nodes to present the translated viral antigens to naive T and B cells.

Simultaneously, the immunological profile of the vaccine is finely tuned through nucleoside modifications and structural optimization of the mRNA itself. While unmodified *in vitro* transcribed (IVT) mRNA can trigger excessive innate sensing via endosomal Toll-like receptors (TLR7/8) and cytosolic sensors like Retinoic acid-inducible gene I (RIG-I), the incorporation of modified nucleosides such as 1-methylpseudouridine minimizes over-activation of Type I IFN pathways (Karikó et al., 2005; Pardi et al., 2018b; Sahin et al., 2014). This modulation prevents the premature shutdown of protein translation, ensuring sustained antigen production while still providing sufficient inflammatory signals to drive a potent, Th1-biased cellular response and high-titer neutralizing antibody production (Pardi et al., 2018b; Teijaro & Farber, 2021). Consequently, the success of LNP-mRNA formulations lies in this delicate

balance: maintaining enough innate stimulation to drive adjuvant activity while preventing the excessive immunogenicity that would otherwise compromise the stability and translational efficiency of the mRNA transcript.



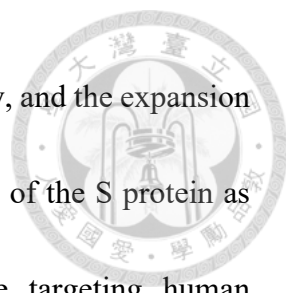
While next-generation LNP-mRNA formulations are demonstrated highly effective at driving systemic IgG production and potent T follicular helper (Tfh) cell responses, achieving robust mucosal immunity, characterized by the secretion of antigen-specific sIgA, via conventional intramuscular (IM) administration remains a significant hurdle (Wu et al., 2024). Recent studies indicate that although IM mRNA-LNPs induce high systemic antibody titers, circulating IgA responses are generally low without prior mucosal priming (Alameh et al., 2024; Alameh et al., 2021). The unique physicochemical properties and intrinsic adjuvant activity of ionizable lipid-based LNPs provide a powerful immunological foundation for optimal B cell activation. First, the potent pro-inflammatory milieu triggered by LNPs at the injection site and draining lymph nodes stimulates the localized expression of critical cytokines, most notably interleukin-6 (IL-6) (Alameh et al., 2021). This LNP-induced IL-6 is essential for early Tfh cell differentiation, which subsequently drives robust germinal center (GC) reactions, providing the necessary signals for the affinity maturation of B cells (Alameh et al., 2021).



To achieve comprehensive lactogenic and mucosal protection, however, this potent GC response must be effectively coupled with the gut-mammary-sIgA axis (Langel et al., 2020). In this highly specialized anatomical network, antigen-specific IgA-committed plasmablasts must be imprinted with mucosal homing markers, such as $\alpha 4\beta 7$ integrin and the chemokine receptor CCR9, allowing them to extravagate and seed distant mucosal effector sites (Langel et al., 2020). Once resident in the lamina propria of the gastrointestinal tract or the vascular endothelium of the lactating mammary gland, these long-lived plasma cells facilitate the continuous, high-titer secretion of dimeric sIgA into colostrum and milk. Therefore, maximizing the efficacy of PEDV mRNA-LNP vaccines requires targeted strategies that not only harness the LNP-driven IL-6/Tfh axis but actively orchestrate the precise mucosal homing of these high-affinity B cells.

1.4.6 Current progress in coronaviral mRNA vaccine research

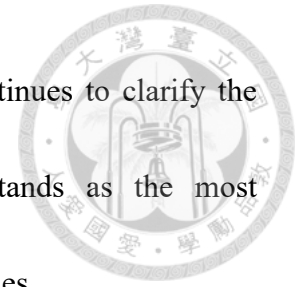
The rapid escalation of the COVID-19 pandemic catalyzed an unprecedented leap in vaccinology, transitioning mRNA technology from an experimental tool to a global clinical standard. The success of the SARS-CoV-2 vaccines, developed by Pfizer-BioNTech and Moderna, provided a definitive proof-of-concept for the safety, scalability, and high efficacy of the LNP-mRNA platform (Chaudhary et al., 2021; Teijaro & Farber, 2021). Current research in coronaviral mRNA vaccines is now focused on refining these



tools to address the challenges of viral evolution, long-term immunity, and the expansion into veterinary medicine. Central to these efforts is the optimization of the S protein as the primary immunogen. Most current designs, including those targeting human coronaviruses and veterinary pathogens, utilize the Receptor Binding Domain (RBD) or the prefusion-stabilized full-length S protein to elicit potent neutralizing antibodies while minimizing the risk of vaccine-associated enhanced respiratory disease (VAERD) and immunopathology (Corbett et al., 2020).

Recent progress in the field has shifted toward the development of multivalent and pan-coronavirus vaccines. These next-generation formulations aim to provide broader protection against emerging variants by encoding conserved epitopes or multiple S protein sequences within a single LNP carrier (Chaudhary et al., 2021; Teijaro & Farber, 2021). Furthermore, significant engineering efforts are being directed toward improving the thermostability of LNP-mRNA formulations, which remains a logistical hurdle for global distribution and on-farm veterinary applications (Chaudhary et al., 2021). In the porcine industry, where PEDV remains a significant economic threat, the modularity of mRNA technology allows for rapid strain-matching, enabling the production of vaccines that can be updated as quickly as new viral lineages emerge (Chaudhary et al., 2021; Pardi et al., 2018a). This transition from human healthcare to animal health represents a critical frontier, where the high-avidity immune responses observed in human trials are being

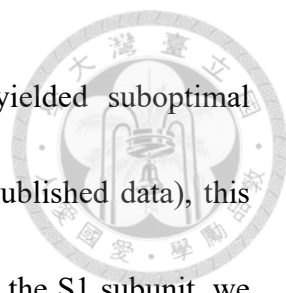
translated to protect the global swine population. As research continues to clarify the complex host-pathogen interactions, LNP-mRNA technology stands as the most adaptable cornerstone for future pandemic preparedness across species.



1.5 Aims of study

The primary objective of this study is to bridge the gap in our fundamental understanding of viral docking and host interaction by characterizing the molecular interplay between the PEDV S protein and a panel of porcine host lectins. In this study, we investigated the role of porcine lectins as potential attachment factors of PEDV. Using a high-throughput lectin ELISA-based screening, candidate lectins, including mKCR, Nkp44, Siglec-9, and TLT2, that exhibit significant binding reactivity toward the recombinant PEDV S glycoprotein were identified. The ectodomains of these porcine lectins were subsequently constructed and expressed in the Madin-Darby canine kidney (MDCK) cell line, which was characterized as none PEDV permissive. After PEDV infection, quantitative RT-qPCR and immunocytochemical staining were performed and no detectable viral infection was noted in these lectins-expressing cells.

In addition to investigate the mechanistic insights of the host-pathogen interactions, this study also aims to develop an optimized preventive strategy against PEDV by next-generation LNP-mRNA vaccine platform. While previous research within our laboratory

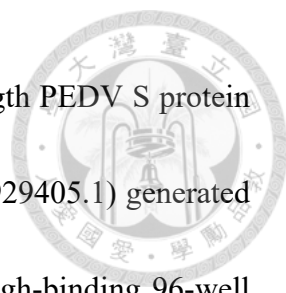


utilizing the full-length S protein as the mRNA immunogen yielded suboptimal immunological outcomes in animal models (Wu et al., (2024), unpublished data), this study pivots to the S1 subunit as the primary target. By focusing on the S1 subunit, we aim to evaluate its superior expression levels observed in preliminary *in vitro* assays and its potential for significantly reduced production costs and cellular metabolic burden compared to the full-length counterpart. This research involves the formulation of a nucleoside-modified mRNA encoding the PEDV S1 subunit delivered via LNPs to establish a robust platform for intracellular translation. The biological performance of this formulation will be assessed to confirm sustained protein expression and functional stability over a three-month duration. Ultimately, this aim seeks to validate the LNP PEDV S1-based mRNA system as a high-potential vaccine candidate, offering a more efficient and cost-effective solution for controlling coronaviral outbreaks in the global swine industry.

Chapter 2. Materials and Methods

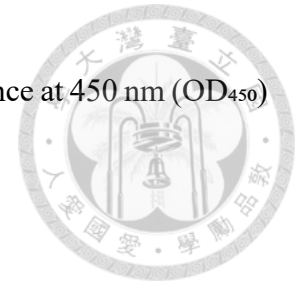
2.1 Evaluation of the Role of Porcine Lectins of mKCR, Nkp44, Siglec-9, and TLT2 in Porcine Epidemic Diarrhea Virus Infection

2.1.1 Lectin binding assay



Lectin ELISA assays were performed as follows. The full-length PEDV S protein of the Taiwan PEDV-Pintung-52 (PEDV-PT) strain (GenBank: KY929405.1) generated from our previous study (Chang et al., 2018) in PBS was used. High-binding 96-well plates were coated overnight at 4 °C with 2.5 µg/mL PEDV S protein (100 µL/well). Plates were washed three times with TBS buffer and blocked with 250 µL/well TBS containing 3% (w/v) polyvinyl alcohol (PVA) for 1 h at room temperature (RT). After washing three times with TBS, human IgG Fc-tagged lectins (10 µg/mL), including hlgG1, AICL, BDCA2, BIMLEC, CD69, CLEC1, CLEC2, CLEC5A, CLEC6, CLEC9A, CLL1, DCIR, DC-SIGN, DC-SIGNR, DEC205, DECTIN1, DECTIN2, Endo180, Langerin, MINCLE, mKCR, ML2, MMR, NKG2D, Nkp30, Nkp44, Siglec-11, Siglec-4a, Siglec-5, Siglec-7, Siglec-9, TLR2, TLR4, TLT2, CLEC5C, LOX1, MAFA, CLEC5B, LSECtin, ASGR-1, mCLEC2L, ASGR-2, CLEC2D, KLRF2, CLEC2A, diluted in lectin-binding buffer (1% BSA, 0.5× Carbo-Free Blocking Solution, TBS, 2 mM MgCl₂, 2 mM CaCl₂) were added (100 µL/well) and incubated at RT for 1.5 h. Plates were washed four times with TBS buffer. Detection was achieved by adding 100 µL/well of HRP-conjugated goat anti-human IgG (1:10,000 dilution in 2% BSA/TBS), followed by incubation at RT for 30 min and six washes with TBS. 100 µL/well of TMB (3,3',5,5'-Tetramethylbenzidine) substrate was then added and incubated for 15 min at RT. The

reaction was stopped by adding 100 μ L/well 2 N H_2SO_4 , and absorbance at 450 nm (OD_{450}) was measured using a microplate reader.



2.1.2 Sequence homology screening, porcine lectin genes identification, and plasmid construction

To ensure the successful generation of expression constructs tailored for use in the porcine system, the initial step involved the precise identification of the porcine homologous gene sequences corresponding to the candidate human lectins, we employed the BLAST (Basic Local Alignment Search Tool) algorithm by aligning the human lectin protein sequences against the protein database within the pig (*Sus scrofa*) genome. Porcine homologous CLEC4F (mKCR), NCR2 (Nkp44), CD33-like (Siglec-9), and TREML2 (TLT2) sequences within the *Sus scrofa* genome exhibiting 75.41%, 77.46%, 77.88%, and 82.71% identity to the corresponding human sequences (Table 1).

The gene sequences corresponding to porcine mKCR, Nkp44, Siglec-9, and TLT2 were synthesized and codon optimized for porcine expression system (Genscript Corp., USA). The synthetic lectin gene fragments were digested using the NotI (New England Biolabs, USA, Cat.: #R3189S) and BamHI (New England Biolabs, USA, Cat.: R3136S) restriction enzymes and subsequently cloned into the pcDNATM3.1/V5-His TOPO[®] vector (Invitrogen, ThermoFisher Scientific, USA) according to the manufacturer's

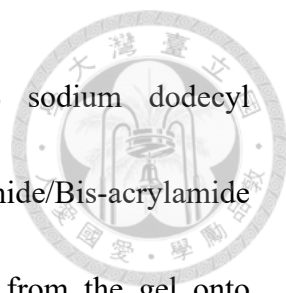
instructions, resulting in the intermediate plasmids, pcDNA3.1A/mKCR/V5-His, pcDNA3.1A/Nkp44/V5-His, pcDNA3.1A/Siglec-9/V5-His, and pcDNA3.1A/TLT2/V5-His (Fig. 2). The entire sequence was confirmed by sequencing conducted by Tri-I Biotech Inc. (Taipei, Taiwan).

2.1.3 Plasmid transfection and expression

To establish lectins-expressing cell lines, the pcDNA3.1A/mKCR/V5-His, pcDNA3.1A/Nkp44/V5-His, pcDNA3.1A/Siglec-9/V5-His, and pcDNA3.1A/TLT2/V5-His plasmids were transfected into HEK293 (PEDV-permissive) and MDCK (PEDV-non-permissive) cells. The transfection procedure was performed using the PolyJet™ *in vitro* DNA transfection reagent (SignaGen® Laboratories, USA) in accordance with the manufacturer's protocols. At 48 hours post-transfection, the cells were fixed with 80% iced-cold acetone or lysed by RIPA lysis and extraction buffer (Thermo Scientific™, USA, Cat.: 89900) following the manufacturer's instruction. The expression of lectins were confirmed by Western blot (WB) and immunocytochemistry (ICC) analysis.

2.1.4 Western blot

Cell lysates were prepared for protein electrophoresis by mixing with 10X NuPAGE® Reducing Agent (Invitrogen, USA) and 4X NuPAGE® LDS Sample Buffer (Invitrogen, USA), followed by denaturation via heating for 5 minutes at 95 °C. The



proteins were then separated by electrophoresis on a 12% sodium dodecyl sulfatepolyacrylamide (SDS) separating gel utilizing a 4% acrylamide/Bis-acrylamide stacking gel. Following separation, the proteins were transferred from the gel onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA) using a BIO-RAD Mini Trans-Blot Electrophoretic Transfer Cell running at 60V for two hours. The membrane was subsequently blocked for one hour in 5% skim milk (Difco™, France) diluted in Tris-buffered saline containing 0.05% Tween 20 (TBST) (Genestar Biotech Inc, Taiwan). For specific detection of the target protein, the membrane was incubated with the primary antibody, anti-V5 Tag Mouse Monoclonal Antibody (Invitrogen, USA #R960-25), diluted 1:5000 in 5% skim milk in Tris-buffered saline with Tween 20 (TBST) (Genestar Biotech Inc, Taiwan) for one hour at room temperature. After six washes with TBST, the membrane was incubated with the secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, USA), diluted 1:10000 in 5% skim milk in TBST for one hour at room temperature. Following every incubation step, the membrane was washed three times with TBST (Genestar Biotech Inc, Taiwan) for 5 minutes at room temperature. The signal was then generated by applying Clarity™ Western ECL Blotting Substrates (Bio-Rad, USA) and detected using ChemiDoc™ Imaging Systems (Bio-Rad, USA).

2.1.5 Immunocytochemistry (ICC) staining



To detect the cellular expression of the lectin proteins, cells transfected with lectin plasmids were first fixed with 80% iced-cold acetone at -20 °C for 30 minutes. After the acetone was discarded and the cells were air-dried overnight. The fixed cells were probed using the primary antibody, V5 tag Monoclonal antibody (Invitrogen, USA, Cat.: R960-25), diluted at 1: 1000 in phosphate-buffered saline (PBS) (Gibco, ThermoFisher Scientific, USA). Incubation with the primary antibody was carried out for one hour at room temperature. Following the primary incubation, cells were washed four to six times with PBS. Subsequently, they were incubated for another one hour at room temperature with the secondary antibody, HRP-conjugated monoclonal anti-rabbit/mouse immunoglobulins (Dako, USA). After six additional washes with PBS, protein visualization was performed using the EnVision-DAB+ system (Dako, USA), strictly adhering to the manufacturer's instructions. Untreated cells served as the negative control. Positive protein expression was indicated by brown signals visible under an inverted light microscope (Nikon, Japan).

2.1.6 Viral propagation and cell culture

The PEDV Pingtung 52 passage 6 (PEDVPT-P6) strain (Genbank Accession No. KY929405) was utilized as the primary seed stock to generate the seventh-passage viral stock (PEDVPT-P7) using Vero C1008 cells (ATCC® CRL-1586™). After freezing and thawing, the harvested viral supernatant was centrifuged at 3000 rpm for 30 minutes to



remove the cell debris and then filtered through 0.22µm Millipore Express® PLUS Polyethersulfone (PES) Membrane syringe filter (Millipore, Merck KGaA, Germany). The cultivation was facilitated by TPA medium, a specialized formulation derived from a Dulbecco's Modified Eagle Medium (DMEM) base (Gibco, ThermoFisher Scientific, USA containing 0.3% tryptose phosphate broth (Sigma-Aldrich, USA), 0.02% yeast extract (Acumedia, USA), and 10 µg/mL trypsin (Gibco, ThermoFisher Scientific, USA) were used.

For the purpose of *in vitro* experimentation, human embryonic kidney (HEK) 293 cells (ATCC® CRL-1537™) and Madin-Darby canine kidney (MDCK) cell (ATCC® CCL-34™) cells were maintained in a growth medium comprising Dulbecco's Modified Eagle Medium (DMEM) (Gibco, ThermoFisher Scientific, USA) supplemented with 10% Fetal Bovine Serum (FBS) (ThermoFisher Scientific, USA) and 1% Antibiotic-Antimycotic (ThermoFisher Scientific, USA).

2.1.7 Viral infection and *in vitro* challenge assays

After 24 hours of transfection, the initial growth medium was aspirated, and the cell monolayers were washed to remove residual serum. The cells were inoculated with PEDV passage 7 (PEDVPT-P7) diluted 1:100 in TPA medium. The plates were incubated for 1 hour to allow for viral entry. Subsequently, the inoculum was removed, and the cells were washed twice with dPBS (Gibco, ThermoFisher Scientific, USA) to eliminate unbound



viral particles. The wells were then replenished with a full volume of fresh TPA medium to support viral replication. The progression of the infection was monitored for 24 to 48 hours, specifically observing the development of cytopathic effects (CPE) and characterizing the extent of viral-induced cellular damage. To ensure mathematical rigor and validate the data for robust statistical significance, all viral infection assays and subsequent quantifications were independently performed in triplicate.

2.1.8 Viral RNA extraction and sample processing

Viral RNA was isolated from infected cells using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) in accordance with the manufacturer's protocols. For high-throughput requirements, large-scale RNA extraction was executed via the QIAcube HT automated system (Qiagen, USA) utilizing the QIAamp cadzor Pathogen Mini Kit (Qiagen, USA) following standardized manufacturer instructions.

2.1.9 Reverse transcription and real-time quantitative RT-PCR analysis

The extracted RNA was subsequently converted into complementary DNA (cDNA) through reverse transcription using the QuantiNova Probe PCR Kit (Qiagen, USA). For the quantification of viral load, a real-time PCR assay was optimized based on established methodologies (Jung et al., 2014), targeting the PEDV-N gene. The reaction utilized a specific fluorogenic probe (5'-FAM-TGYTACCAYYACCACGACTCCTGC-BHQ1-3')

in conjunction with the following primer set: Forward Primer: 5'-CGCAAAGACTGAACCACTAAC-3'; Reverse Primer: 5'-TTGCCTCTGTTGTTACTTGGAGAT-3'.



2.1.10 Immunocytochemistry staining for PEDV detection

To detect the PEDV infection, cells infected with PEDV at 48 hours post-infection were fixed with 80% iced-cold acetone at -20 °C for 30 minutes. After the acetone was discarded and the cells were air-dried overnight. The fixed cells were probed using the primary antibody, homemade porcine P4B antibody (Chang et al., 2019), diluted at 1: 200 in PBS (Gibco, ThermoFisher Scientific, USA). Incubation with the primary antibody was carried out for one hour at room temperature. Following the primary incubation, cells were washed four to six times with PBS. Subsequently, they were incubated for another one hour at room temperature with the secondary HRP-conjugated monoclonal anti-rabbit/mouse immunoglobulins (Dako, USA). After six additional washes with PBS, protein visualization was performed using the EnVision-DAB+ system (Dako, USA), strictly adhering to the manufacturer's instructions. Untreated cells served as the negative control. Positive protein expression was indicated by brown signals visible under an inverted light microscope (Nikon, Japan).

2.2 Development of Lipid Nanoparticle S1-based mRNA Vaccine Against Porcine Epidemic Diarrhea Virus

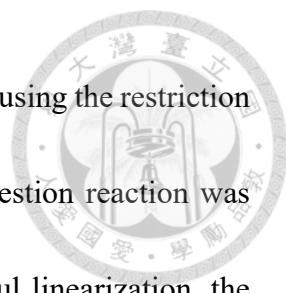
2.2.1 Preparation and construction of plasmid pcDNA3.1A/PEDV S1/V5-His

The base genetic material, encoding the ectodomain of the PEDV S1, was initially carried on the pcDNA3.1ATM PEDV S1/V5-His plasmid. This specific PEDV S1 sequence was derived from the Taiwan G2b Pintung 52 (PEDV-PT) strain (GenBank accession number: KP276252), which belongs to the genotype IIb (G2b) PEDV. Crucially, the gene sequence included a tissue plasminogen activator signal peptide (tPA-SP) sequence to direct protein secretion (Chang et al., 2018).

A synthetic gene segment was custom-ordered from Genscript Corporation (Piscataway, USA) for insertion into the vector backbone. This segment consisted of a continuous sequence of 120 adenines (designated as poly(A) 120), internally separated by six guanines into two equal segments of 60 adenines each (Trepotec et al., 2019).

The synthetic gene fragment was excised using the NotI and PmeI restriction enzymes. This digested fragment was subsequently cloned into the pcDNATM3.1/V5-His TOPO® vector, resulting in the intermediate plasmid, pcDNA3.1A/V5-His. The final recombinant plasmid, pcDNA3.1A/PEDV S1/V5-His, was generated by combining the necessary elements. The clones of the resultant plasmid were critically checked for correct insertion and sequence fidelity. The entire sequence was confirmed for accuracy via nucleotide sequencing conducted by Tri-I Biotech Inc. (Taipei, Taiwan).

2.2.2 Generation of messenger RNA



The pcDNA3.1A/PEDV S1/V5-His plasmid was first linearized using the restriction enzyme PmeI (New England Biolabs, USA, Cat.: R0560). The digestion reaction was carried out at 37 °C for a duration of one hour. To verify successful linearization, the resulting digested plasmid was subjected to analysis via 0.8% agarose gel DNA electrophoresis. Subsequently, the desired linear DNA fragment was isolated and purified from the gel matrix utilizing the Qiagen QIAquick gel extraction kit (Qiagen, Holland). This purification step ensured a high-quality product following the initial construction phase.

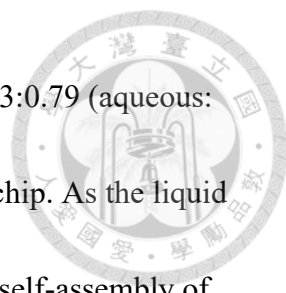
Concurrently, a laboratory-stock pcDNA3.1A/GP5/V5-His plasmid was utilized as a functional positive control, subjected to the identical linearization and gel purification workflows to benchmark downstream assay performance. *In vitro* transcription was executed following the manufacturer's protocols using the RiboMAX™ Large Scale RNA Production System (Promega, USA). To ensure the production of translationally competent mRNA, the reaction mixture was supplemented with a 5'-cap analog (m7G(5')ppp(5')G) (Invitrogen, USA, Cat.: AM8050). The synthetic procedure was carried out at 37 °C for four hours. Following transcription, the resulting mRNA was purified via lithium chloride (LiCl) (Invitrogen, USA, Cat.: AM9480) precipitation. Specifically, the mRNA was combined with 2.5 mM LiCl and incubated at -20 °C for a minimum of 30 minutes to promote selective precipitation. The precipitate was pelleted

by centrifugation at 13,000 rpm at 4 °C for 10 minutes. The resulting mRNA pellet was subsequently washed once with cold 70% ethanol and subjected to a second centrifugation step 13000 rpm at 4 °C for 5 minutes. The purified mRNA pellet was finally resuspended in nuclease-free water, its final concentration was measured, and the sample was preserved at -80 °C for long-term storage.

2.2.3 Formulation of lipid nanoparticles (LNPs)

The lipid components consisted of SM-102 (20 mg/mL), DOPE (8 mg/mL), DMG-PEG-2000 (20 mg/mL), and cholesterol (6 mg/mL), which were precisely measured and dissolved in ethanol. The mixture consisted of these four components at specific molar ratios of 35:16:2.5:46.5 (corresponding to ionizable lipid (SM102) (MedChemExpress, USA, Cat.: 190279): dioleoylphosphatidylethanolamine (DOPE) (Avanti, USA, Cat.: 850725P-500MG-A-441): 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG-2000) (Avanti, USA, Cat.: 880151P-1G-A-022): cholesterol (Sigma-Aldrich, USA, Cat.: SLCG8406), respectively) to a total lipid volume of 320 μ L per reaction. After dissolution in ethanol, the lipid mixture was sonicated to ensure uniformity and filtered through a 0.22 μ m syringe filter (Merck Millipore Ltd.).

For LNP formation, purified mRNA (100 μ g) was diluted in 1230 μ L of 65 mM sodium citrate buffer (pH 4.0). This aqueous mRNA solution was rapidly mixed with



the filtered lipid mixture (ethanol phase) at a precise volume ratio of 3:0.79 (aqueous: ethanol) using syringe pumps slowly fed into a custom microfluidic chip. As the liquid was manually expelled, the rapid and homogenous mixing drove the self-assembly of uniform LNPs encapsulating the mRNA molecules. Following formulation collection, the mixtures were diluted 3.25-fold with 65mM citrate buffer, incubated at room temperature for 30 minutes, and subsequently dialyzed against PBS (> 50X) using 10 kDa molecular weight cutoff (MWCO) tubes (Sigma-Aldrich) via centrifugation at 2000 x g at 4 °C for 1-2 hours. The dialyzed LNP-mRNA formulations were again passed through a 0.22µm filter, and the encapsulation efficiency (EE) was quantified utilizing the RiboGreen assay (Invitrogen). The finalized formulations were stored at 4 °C for downstream immunization studies.

2.2.4 Evaluation of mRNA transfection efficiency

To assess the expression of the target protein, 5 µg of LNP-encapsulated PEDV S1 mRNA or LNP-encapsulated control mRNA was directly added into the culture medium of 10⁶ HEK293 cells per well in a 6-well plate. For comparison, 5 µg of the same *in vitro* transcribed mRNA was transfected into a parallel set of cells under identical culture conditions using the commercial transfection reagent, LipofectamineTM MessengerMAX (ThermoFisher Scientific, USA), following the manufacturer's recommended protocol.

This parallel setup facilitates the comparative evaluation of the expression efficiency achieved by the LNP formulation against those of naked mRNA.



2.2.5 Immunocytochemistry (ICC) staining

To detect the cellular expression of the PEDV S1 protein, HEK293 cells transfected with either LNP PEDV S1 mRNA or PEDV S1 mRNA complexed with Lipofectamine™ MessengerMAX transfection reagent were processed 24 hours post-treatment. Cells transfected with the GP5 mRNA derived from the pcDNA3.1A/GP5/V5-His plasmid were processed in parallel from the onset of the IVT phase, served as the experimental positive control.

The cells were first fixed with 80% iced-cold acetone at -20 °C for 30 minutes. After the acetone was discarded and the cells were air-dried overnight, the fixed cells were probed using the primary antibody, V5 tag monoclonal antibody (Invitrogen, USA, Cat.: R960-25), diluted at 1: 1000 in Phosphate-Buffered Saline (PBS) (Gibco, ThermoFisher Scientific, USA). Incubation with the primary antibody was carried out for one hour at room temperature. Following the primary incubation, cells were washed four to six times with PBS. Subsequently, they were incubated for another one hour at room temperature with the secondary antibody, monoclonal anti-rabbit/mouse immunoglobulins conjugated with horseradish peroxidase (HRP) (Dako, USA). After four to six additional washes with PBS, protein visualization was performed using the

EnVision-DAB⁺ system (Dako, USA), strictly following the manufacturer's instructions.

Untreated HEK293 cells served as the negative control. Positive protein expression was indicated by brown signals visible under an inverted light microscope (Nikon, Japan).

2.2.6 Western blot

To determine the presence and size of the expressed protein, HEK293 cells treated with LNP-mRNAs or mRNA-Lipofectamine[™] MessengerMAX were harvested at 24 hours post-treatment and lysed using RIPA lysis buffer (Energogenesis Biomedical Co, Ltd, Taiwan Cat.: RP05-10). Cells transfected with the GP5 mRNA derived from the pcDNA3.1A/GP5/V5-His plasmid were processed in parallel from the onset of the IVT phase, served as the experimental positive control.

The resulting cell lysates were prepared for protein electrophoresis by mixing with 10X NuPAGE[®] Reducing Agent (Invitrogen, USA) and 4X NuPAGE[®] LDS Sample Buffer (Invitrogen, USA), followed by denaturation via heating for 5 minutes at 95 °C. The proteins were then separated by electrophoresis on a 12% sodium dodecyl sulfatepolyacrylamide (SDS) separating gel utilizing a 4% acrylamide/Bis-acrylamide stacking gel. Following separation, the proteins were transferred from the gel onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA) using a BIO-RAD Mini Trans-Blot Electrophoretic Transfer Cell running at 60V for two hours. The membrane was subsequently blocked for one hour in 5% skim milk (Difco[™], France) diluted in Tris-



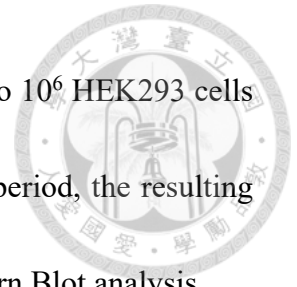
buffered saline containing 0.05% Tween 20 (TBST) (Genestar Biotech Inc, Taiwan). For specific detection of the target protein, the membrane was incubated with the primary antibody, V5 tag mouse monoclonal antibody (Invitrogen, USA #R960-25), diluted 1:5000 in 5% skim milk in TBST for one hour at room temperature. After six washes with TBST (Genestar Biotech Inc, Taiwan), the membrane was incubated with the secondary antibody, HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, USA), diluted 1:10000 in 5% skim milk in TBST for one hour at room temperature. Following every incubation step, the membrane was washed three times with TBST (Genestar Biotech Inc, Taiwan) for 5 minutes at room temperature. The signal was then generated by applying Clarity™ Western ECL Blotting Substrates (Bio-Rad, USA) and detected using ChemiDoc™ Imaging Systems (Bio-Rad, USA).

2.2.7 Long-term stability assessment of LNP-Encapsulated mRNA

To thoroughly evaluate the long-term stability and functional performance of the LNP-encapsulated mRNA, a controlled study was initiated. The LNP mRNA formulation was stored under refrigerated conditions at 4 °C to simulate common pharmaceutical storage requirements. Stability and functional activity were assessed periodically, specifically conducting measurements once a month for a total duration of three months.

At each time point (1st month, 2nd month, and 3rd month), the functional stability was determined by measuring the expression level of the encoded protein following cellular

delivery. For this assay, 5 μ g of the stored LNP mRNA was added to 10^6 HEK293 cells seeded in each well of a 6-well plate. After a 24 hours incubation period, the resulting cellular protein expression was quantitatively measured using Western Blot analysis.



Chapter 3 Results



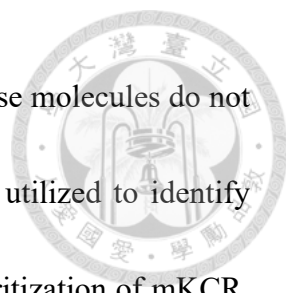
3.1 Evaluation of the Role of Porcine Lectins of mKCR, Nkp44, Siglec-9, and TLT2 in Porcine Epidemic Diarrhea Virus Infection

3.1.1 High-throughput screening of PEDV spike-binding lectins

To systematically identify host carbohydrate-binding proteins that potentially interact with the PEDV, a high-throughput ELISA-based screening was conducted. Using purified recombinant PEDV S protein as the bait, we evaluated its binding affinity against an extensive 45 lectins. The assay identified a specific subset of lectins, including DC-SIGN, DC-SIGNR, Langerin, mKCR, Nkp44, Siglec-9, and TLT2, that exhibited significant binding reactivity toward the S protein (Fig. 1). These findings suggest that the complex N-linked glycans decorating the PEDV S protein serve as specific ligands for multiple host lectins, potentially facilitating viral recognition and attachment during the initial stages of infection.

3.1.2 Generation and evaluation the expression of porcine lectins

Following initial screening, candidate lectins underwent a rigorous selection process for functional characterization. While DC-SIGN, DC-SIGNR, and Langerin exhibited binding reactivity in ELISA assays, they were excluded as previous studies



(Subramaniam et al., 2017; Zhao et al., 2021) have demonstrated these molecules do not function as productive receptors for PEDV infection. BLAST was utilized to identify porcine homologs within the *Sus scrofa* genome, leading to the prioritization of mKCR, Nkp44, Siglec-9, and TLT2 based on binding intensity and physiological relevance to the porcine respiratory and intestinal tracts. Bioinformatic analysis confirmed high sequence conservation in their carbohydrate-recognition domains, specifically identifying the homologs as CLEC4F (mKCR), NCR2 (Nkp44), CD33-like (Siglec-9), and TREML2 (TLT2) (Table 1). These sequences served as the genetic blueprint for pcDNA3.1A-based porcine lectin plasmids, which were subsequently transfected into HEK293 cells. ICC analysis, quantified via ImageJ, detected cytoplasmic expression in 54.8% (mKCR), 43.5% (Nkp44), 51.9% (Siglec-9), and 14.9% (TLT2) of cells (calculated by ImageJ, NIH, USA) (Fig. 3A, left). Furthermore, Western blot analysis identified distinct molecular profiles with bands at approximately 100 kDa (mKCR), 44 kDa (Nkp44), 55 kDa (Siglec-9), and 46 kDa (TLT2) (Fig. 3B). The observed molecular weights were consistently higher than predicted from the primary amino acid sequences, suggesting extensive post-translational modifications.

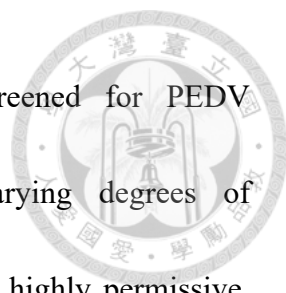
Table 1. Identification and annotation of porcine lectin homologs.



Human	Porcine Homolog	Porcine Gene Annotation	Percent
Lectin	Gene Bank		Identity
	Accession		(compared with human)
mKCR	XM_021087395.1	<i>Sus scrofa</i> C-type lectin domain family 4 member F (CLEC4F)	75.41%
Nkp44	XM_02108670.1	<i>Sus scrofa</i> natural cytotoxicity triggering receptor 2 (NCR2)	77.46%
Siglec-9	XM_021094848.1	<i>Sus scrofa</i> myeloid cell surface antigen CD33-like (LOC110261034)	77.88%
TLT2	XM_021098669.1	<i>Sus scrofa</i> triggering receptor expressed on myeloid cells like 2 (TREML2)	82.71%

3.1.3 Identification of MDCK as a non-permissive model for PEDV infection

To identify a cellular environment with low background susceptibility for validating lectin-mediated entry, a panel of porcine and non-porcine cell lines, including PK-15,



IPEC-J2, ST, BHK-21, 3D4/21, C127, and MDCK, was screened for PEDV permissibility (Table 2). RT-qPCR analysis demonstrated varying degrees of susceptibility. Notably, cell lines such as PK-15 and 3D4/21 were highly permissive, while MDCK cells showed no detectable viral replication (Ct: N/A). Consequently, the MDCK cell line was selected as the definitive non-permissive model to evaluate the potential function of candidate lectins in facilitating viral attachment and entry.

Table 2. The permissibility of cell lines to PEDV Infection.

Cell	Species	Tissue/Cell Type	Mean Ct	Permissivity
Line	Origin		Value	Status
PK-15	Porcine	Kidney epithelial	22.89	Highly Permissive
3D4/21	Porcine	Alveolar macrophage	23.39	Highly Permissive
IPEC-J2	Porcine	Intestinal columnar epithelial	26.27	Moderately Permissive
C127	Murine	Mammary tumor	28.42	Mildly Permissive

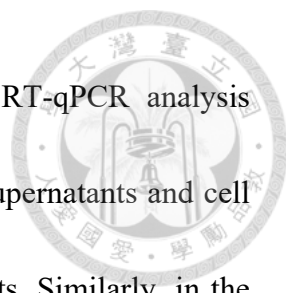


BHK-21	Hamster	Kidney	31.23	Very Mildly
		fibroblast		Permissive
ST	Porcine	Testicular	32.44	Very Mildly
		epithelial		Permissive
MDCK	Canine	Kidney epithelial	N/D	Non-Permissive

Note: Cycle threshold (Ct) values were determined 48 hours post-infection. A lower Ct value indicates a higher viral RNA load. "ND" denotes no detectable amplification after 40 cycles.

3.1.4 Porcine lectins were not able to support PEDV infection in non-permissive MDCK cells quantitative analysis of viral RNA

To evaluate whether candidate porcine lectins function as entry factors for PEDV, mKCR, Nkp44, Siglec-9, and TLT2 were expressed in the non-permissive MDCK model, with cytoplasmic expression verified via ICC, showing rates of 22.7% (mKCR), 18.9% (Nkp44), 16.2% (Siglec-9), and 11.1% (TLT2) (calculated by ImageJ, NIH, USA) (Fig. 3A, right), and Western blot analysis (Fig. 3B). Viral replication kinetics were assessed (Table 3) through a longitudinal sampling strategy at 0, 24, and 48 hours post-infection (hpi), where a stringent dPBS washing protocol post-adsorption ensured a clean baseline of non-detectable viral RNA (Ct: N/A) at 0 hpi. While positive control Vero cells



exhibited robust, time-dependent increases in viral RNA titers, RT-qPCR analysis revealed that viral RNA remained undetectable (Ct: N/A) in both supernatants and cell lysates of naïve and lectin-expressing MDCK cells at all time points. Similarly, in the naturally permissive HEK293 model (Table 4), expression of these candidate lectins did not alter viral loads compared to the blank control group. These quantitative findings were further corroborated by ICC staining at 48 hpi (Fig. 4), which detected the PEDV N protein exclusively in Vero cells, while all MDCK experimental groups exhibited a complete absence of viral antigens. Collectively, the lack of intracellular viral RNA and protein expression demonstrates that these candidate lectins do not function as independent entry receptors for PEDV in the models tested.

Table 3. Real-time qPCR quantification of average viral load (log/mL) in candidate lectin-expressing MDCK cells. Note: "ND" denotes no detectable viral RNA amplification after 40 cycles of PCR, confirming the non-permissive status of MDCK cells.

	Supernatant			Cell lysate
	0 hpi	24 hpi	48 hpi	48 hpi
MOCK Vero	N/D	6.97±0.26	8.16±0.08	11.06±0.08
MOCK MDCK	N/D	N/D	N/D	N/D
MDCK mKCR	N/D	N/D	N/D	N/D
MDCK Nkp44	N/D	N/D	N/D	N/D
MDCK Siglec-9	N/D	N/D	N/D	N/D
MDCK TLT2	N/D	N/D	N/D	N/D

Table 4. Real-time qPCR quantification of viral RNA in cell lysate of transfected

HEK293 cells at 48hpi. Note: Data are presented as mean viral load (log/mL). No

significant differences in viral RNA levels were observed between the control and lectin-

transfected groups, suggesting that these candidates do not independently enhance the

innate susceptibility of HEK293 cells to PEDV.

Cell	MOCK	MOCK	HEK	HEK	HEK	HEK
	Vero	HEK	mKCR	Nkp44	Siglec-9	TLT2
Viral load	11.11±1.1	10.20±0.4	10.25±0.45	9.45±0.1	9.14±1.67	9.88±1.10

3.2 Development of Lipid Nanoparticle S1-based mRNA Vaccine Against Porcine

Epidemic Diarrhea Virus

3.2.1 Generation of lipid nanoparticles-mRNA

To generate a suitable DNA template for *in vitro* transcription, the recombinant plasmid pcDNA3.1/PEDV S1/V5-His was successfully linearized using restriction enzyme digestion. Agarose gel electrophoresis confirmed a single, distinct DNA band of approx.. 7.8 kb, corresponding to the expected size of the linearized construct (Fig. 5).

This linearized construct served as the primary template for *in vitro* transcription, which was followed by the LNP encapsulation process to yield the functional complex PEDV S1 mRNA. The encapsulation efficiency was confirmed to be highly effective, demonstrating greater than 80% encapsulation and an overall RNA recovery exceeding 50% (Table 5).

Table 5. The LNP-mRNA encapsulation and recovery rate.

mRNA	Concentration	Volume	Encapsulation	RNA
	($\mu\text{g/mL}$)	(mL)	Efficiency (%)	Recovery (%)
PEDV S1	102.04	0.5	84.52	51.02

3.2.2 Assessment of mRNA delivery and target protein expression

To evaluate the functional efficacy of the LNP mRNA mediating the expression of target proteins within the host cells, both ICC and WB were systematically performed. Qualitative assessment via ICC, utilizing an anti-V5 antibody for detection, revealed that all LNP mRNA successfully delivered their cargoes, as evidenced by the presence of diffusely positive protein signals localized within the cytoplasm of the treated HEK293 cells (Fig. 6). For quantitative size verification, WB analysis confirmed the successful expression and molecular weights of the PEDV S1 protein (Fig. 7). The detected protein

sizes were determined to be approximately 100 kDa for PEDV S1 in the corresponding LNP mRNA treated cells.



3.2.3 Long-term stability and functional efficacy of LNP mRNAs

A long-term study was conducted to investigate the stability profiles of the LNP PEDV S1 mRNA. The formulation was maintained under refrigerated conditions at 4 °C for up to 3 months. The results demonstrated that the LNP PEDV S1 mRNA retained its functional activity, successfully transfecting and expressing PEDV S1 protein in HEK293 cells even after storage at 4 °C for 3 months, indicating the successful maintenance of a stable and consistent level of protein expression over the entire storage period (Fig. 8).

Chapter 4. Discussion

The candidate receptors identified in this study, including mKCR, Nkp44, Siglec-9, and TLT2, belong to a diverse array of innate immune receptor families. Rather than sharing a single structural motif like a CRD, these receptors engage pathogens through highly reciprocal glycan-protein interactions at the virus-host interface. Specifically, while true host lectins actively target the dense glycan shield decorating viral envelope proteins, other Immunoglobulin (Ig)-like host receptors provide heavily glycosylated docking sites for viral lectins.

The binding specificity is determined by the distinct structural and carbohydrate preferences of each interacting pair. For example, Siglec-9, an I-type lectin, is known for its ability to bind the terminal sialic acid residues of the HIV-1 gp120 glycoprotein, a mechanism the virus exploits to facilitate viral adhesion and enhance productive infection (Zou et al., 2011). Similarly, C-type lectin-like receptors such as CLEC5A (relative to mKCR) directly interact with the glycoproteins of viruses like Dengue, which triggers massive inflammatory cascades (Chen et al., 2008).

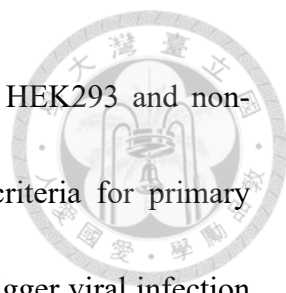
In a highly reciprocal manner, the Ig-like receptor Nkp44 serves as a docking site for the hemagglutinin (HA) of Influenza A Virus, where the viral HA specifically targets terminal sialylated glycans presented on the host receptor (Ho et al., 2008). Furthermore, Nkp44 directly interacts with the envelope (E) proteins of Dengue and West Nile viruses



via its extracellular Ig-domain (HersHKovitz et al., 2009). While the specific viral ligands for the Ig-like receptor TLT2 have been historically elusive, its close relative TREM-1 interacts directly with the heavily glycosylated glycoproteins of Ebola and Marburg viruses through its extracellular Ig-like domain (MohamadZadeh et al., 2006).

The strength of these interactions is driven by multivalent avidity (the velcro effect), where multiple host receptors cluster together to bind numerous sites on the viral spike protein simultaneously. This reciprocal dual-binding strategy, where host lectins target viral glycans and viral lectins target host glycans, combined with our current findings, suggests that these receptors possess a conserved functional affinity for the specific surface landscapes of high-pathogenicity viruses, allowing them to serve as stable anchoring points on the host plasma membrane.

In the hierarchy of viral entry, a functional receptor or co-receptor must not only facilitate physical attachment but also actively participate in triggering conformational changes in the viral fusion machinery. While proteins like pAPN were historically classified as definitive receptors for PEDV, their recent demotion to non-essential factors suggests a more complex, multi-component entry process (Li et al., 2017). A true co-receptor typically acts in concert with a primary receptor to mediate membrane fusion or endocytic internalization (Van Breedam et al., 2014). However, our observation that mKCR, Nkp44, Siglec-9, and TLT2 facilitate robust binding to the PEDV S protein



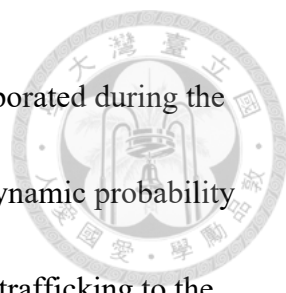
without conferring susceptibility to infection in PEDV permissive HEK293 and non-permissive MDCK cells, suggesting their inability to fulfill the criteria for primary functional receptors. Furthermore, their inability to independently trigger viral infection indicates a block at the entry stage, likely due to the absence of specific host-cell proteases or secondary receptor signals required for final activation.

The experimental evidence suggests that mKCR, Nkp44, Siglec-9, and TLT2 function as non-permissive attachment factors. Unlike receptors that initiate the fusion process, attachment factors serve to block virions from the extracellular environment and concentrate them on the plasma membrane, thereby increasing the local avidity of the virus-cell interaction (Van Breedam et al., 2014; Zhao et al., 2021). This sequestration role is a common pathogenic strategy; for instance, DC-SIGN and L-SIGN act as molecular bridges that capture virions and present them to susceptible cells, a process known as *trans*-infection (Van Breedam et al., 2014), while they can also directly mediate *cis*-infection for PEDV (Zhao et al., 2021). In the context of the porcine gut, these candidate lectins may facilitate tissue-specific docking, allowing PEDV to withstand mucosal clearance and increasing the probability of encountering the necessary proteolytic environment or definitive entry factors on target enterocytes (Li. et al., 2016). Thus, these lectins might act as initial tethering phase of the infection cycle.



To further investigate a definitive mechanistic role for mKCR, NKp44, Siglec-9, and TLT2 as authentic virus-host attachment interfaces, it is crucial to reconcile their structural topology with their spatial distribution within the host cell architecture. These candidate immune receptors are structurally characterized as integral transmembrane proteins, which naturally rely on precise hydrophobic α -helical segments to anchor across the phospholipid bilayer and project their diverse extracellular domains, ranging from CRDs to Ig-like domains, into the extracellular space (Van Breedam et al., 2014). In heterologous expression systems or transient transfection models involving non-permissive cells, a frequent biochemical concern is whether the observed non-permissiveness to viral infection stems from a genuine lack of entry-receptor functionality or a catastrophic defect in protein trafficking, wherein the engineered receptors remain trapped within the endoplasmic reticulum (ER) or Golgi apparatus (Aricescu & Owens, 2013).

Crucially, while our ICC analysis successfully confirmed robust total cellular protein expression, the protocol inherently utilizes acetone fixation, which permeabilizes the lipid bilayer and prevents the empirical discrimination between intracellularly retained proteins and those properly inserted into the plasma membrane. To proactively address this structural confounder from a structural vaccinology and design perspective, highly optimized mammalian signal peptides and stringent codon



optimization tailored for the host translational machinery were incorporated during the initial molecular cloning phases, inherently maximizing the thermodynamic probability of successful co-translational translocation and subsequent vesicular trafficking to the cell surface (Gustafsson et al., 2004; Mauro & Chappell, 2014). Consequently, while the complete absence of CPE and viral replication in our challenge assays demonstrates that these candidate molecules do not independently unlock the downstream viral membrane fusion cascade, further investigations employing non-permeabilized surface-labeling techniques will be valuable to fully define their precise membrane translocation dynamics and spatial accessibility at the virus-host interface (Huang et al., 2022; Van Breedam et al., 2014).

In summary, our study clarifies that these roles based on the observation that while these lectins provide the necessary avidity for physical sequestration of the virion on the plasma membrane, they might be insufficient to independently trigger the critical proteolytic activation required for productive infection.

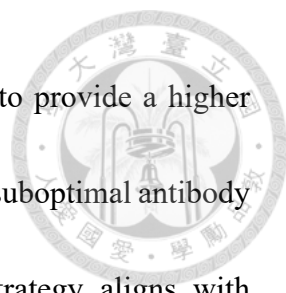
Significantly, this research represents the first experimental investigation to characterize the direct binding interactions between the PEDV S protein and this specific panel of porcine host lectins, mKCR, Nkp44, Siglec-9, and TLT2, addressing a previously unexplored dimension of the viral attachment landscape. Our findings demonstrate that

these lectins might serve as novel attachment factors rather than primary functional receptors.



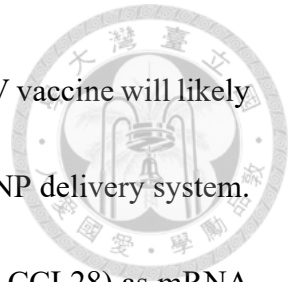
Furthermore, the strategic transition from a full-length S protein to the S1 subunit as the primary vaccine antigen is supported by comparative expression data and previous experimental outcomes within our laboratory. Previous investigations conducted by Wu (Wu et al., 2024, unpublished data) utilized an LNP full-length PEDV S mRNA vaccine. While this vaccine successfully induced systemic IgG and partial clinical protection against viral challenge by two to three vaccine injection, it failed to elicit critical mucosal immunity (IgA) and sterilizing protection. This suboptimal efficacy may be partially attributed to the inherent structural complexity and large molecular weight (approximately 180-200 kDa) of the full-length spike protein, which can impose a significant metabolic burden such as on host cells and lead to inefficient protein folding or rapid degradation.

In contrast, our current Western blot analysis demonstrates that the S1 subunit achieves markedly higher expression levels *in vitro* compared to the full-length S protein (Fig. 9). By focusing exclusively on the S1 domain, which contains the critical NTD, RBD, and primary neutralizing epitopes (such as the COE domain), we concentrate the host's translational machinery on producing the most immunologically relevant



neutralizing epitopes. This increased antigen yield is hypothesized to provide a higher density of targets for the immune system, potentially overcoming the suboptimal antibody responses observed in prior full-S trials. This high-expression strategy aligns with international findings by Makadiya et al. (2016), who demonstrated that the S1 domain alone is capable of inducing potent neutralizing antibodies, although traditional recombinant subunit approaches failed to provide complete clinical protection (Makadiya et al., 2016). While recent studies such as Zhao et al. (2024) have successfully utilized mRNA platforms expressing the full-length spike protein to protect piglets (Zhao et al., 2024), our approach specifically targets the S1 domain to concentrate the immune response on the critical neutralizing epitopes within the N-terminal and receptor-binding domains. Furthermore, as demonstrated by our prior evaluations of this delivery system, the LNP-mRNA platform exhibited sustained functional stability for at least three months at 4 °C, offering a logistically feasible alternative for the swine industry. Compared to the lower efficacy observed in previous recombinant S1 protein trials (Makadiya et al., 2016; Oh et al., 2014), the LNP-mRNA platform's ability to induce robust intracellular antigen expression positions it as a superior candidate for overcoming the historical limitations of PEDV vaccine development. However, while the LNP PEDV S1 mRNA platform exhibited superior expression profiles and functional stability, transitioning to a subunit approach may reduce the overall breadth of T-cell epitopes available compared to the full-

length protein. Therefore, the development of a highly effective PEDV vaccine will likely need the identification of synergistic adjuvants to complement the LNP delivery system. Given that previous trials utilizing specific chemokines (CCL25 and CCL28) as mRNA adjuvants failed to effectively enhance mucosal responses, future studies should focus on alternative immunomodulatory strategies, such as incorporating toll-like receptor (TLR) agonists or exploiting the intrinsic adjuvant activity of the LNP carrier itself (Alameh et al., 2021), to enhance the magnitude and duration of the protective humoral and mucosal immune responses *in vivo*.



Figures

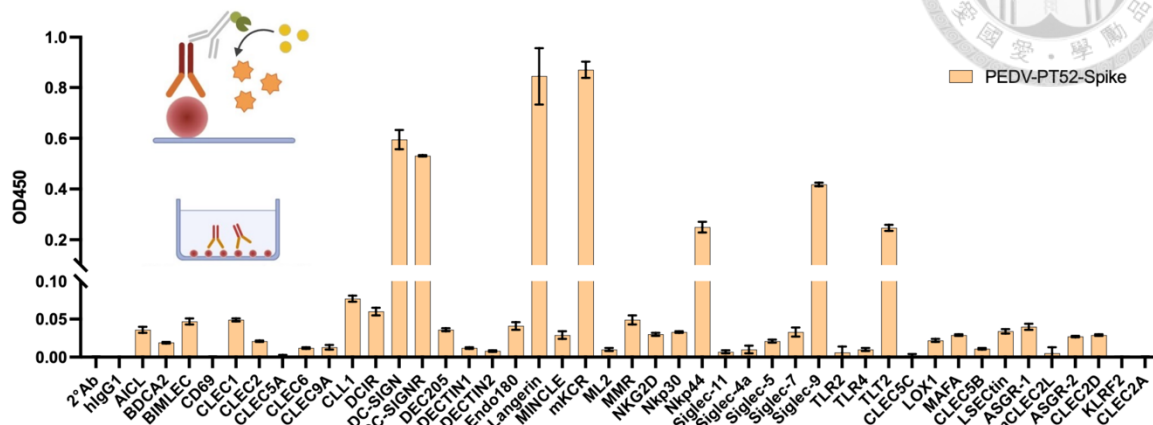


Fig. 1. Identification of affinity binding of host lectins with the PEDV S protein using the high-throughput ELISA-based screening. High-binding 96-well plates were coated with 2.5 $\mu\text{g/mL}$ PEDV S protein (100 $\mu\text{L/well}$) and interacted with human IgG Fc-tagged lectins (10 $\mu\text{g/mL}$). Detection was achieved by adding 100 $\mu\text{L/well}$ of HRP-conjugated goat anti-human IgG (1:10,000 dilution in 2% BSA/TBS), followed by TMB (3,3',5,5'-Tetramethylbenzidine) substrate detection. The absorbance at 450 nm (OD_{450}) was measured using a microplate reader.

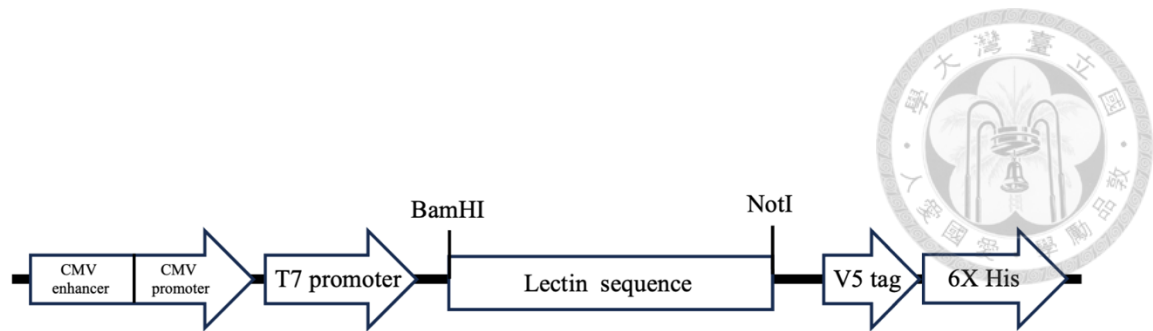


Fig. 2. Construction and enzymatic verification of pcDNA3.1A-based porcine lectin plasmids. Representative vector map showing the arrangement of the Cytomegalovirus (CMV) promoter signal peptide, candidate lectin ectodomain, and V5-His tag, with highlighted NotI and BamHI restriction sites.

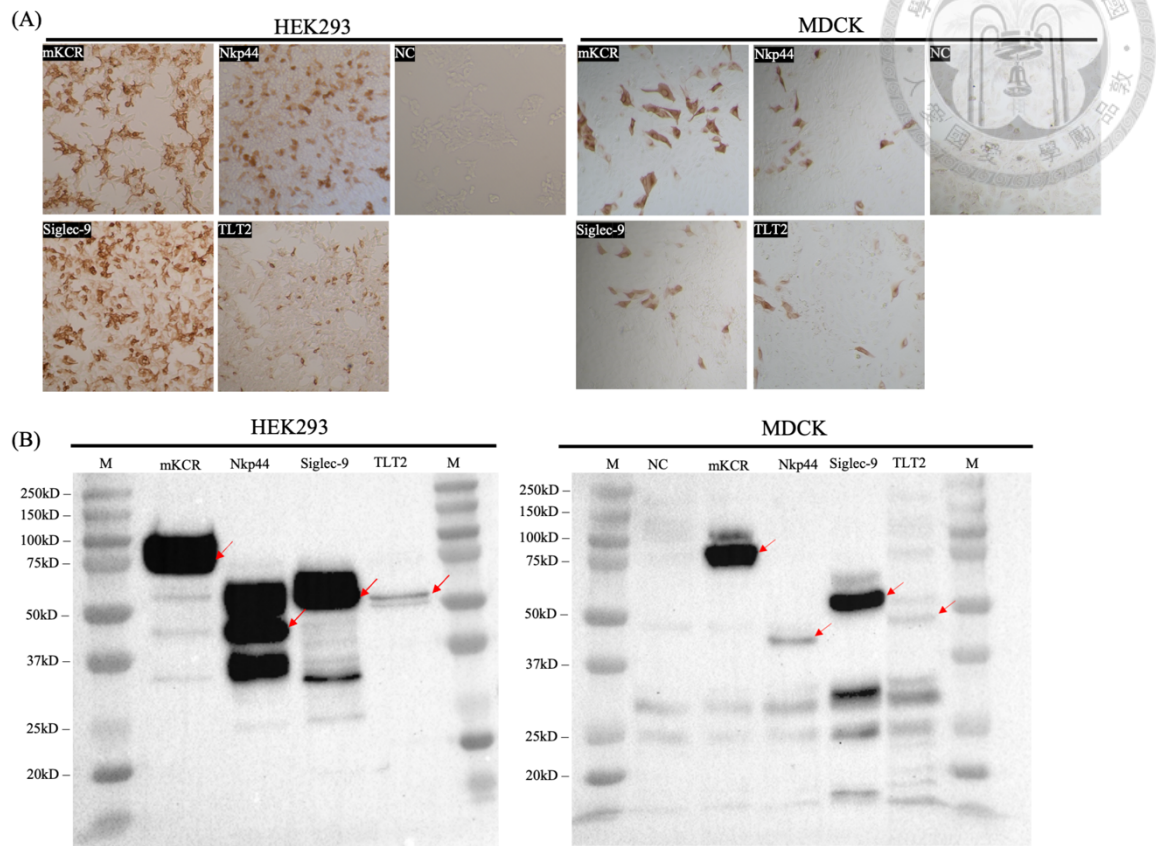


Fig. 3. Detection of recombinant porcine lectins expression in HEK293 and MDCK cells. HEK293 and MDCK cells were transfected by plasmids encoding mKCR, Nkp44, Siglec-9, and TLT2. At 48 hours post-transfection, cells were harvested based on the downstream applications. For ICC, cells were fixed with ice-cold 80% acetone, incubated with a primary anti-V5 tag monoclonal antibody and visualized using the EnVision-DAB+ system. For Western blotting, cells were lysed with RIPA buffer prior to protein analysis with V5-tagging. (A) Representative ICC images showing the cellular localization of V5-tagged lectins (B) Western blot analysis of cell lysates confirming the

molecular weights and expression levels of the recombinant proteins. (M: Marker; NC: Negative Control; mKCR: 100kD; Nkp44: 44kD; Siglec-9: 55kD; TLT2: 46kD)



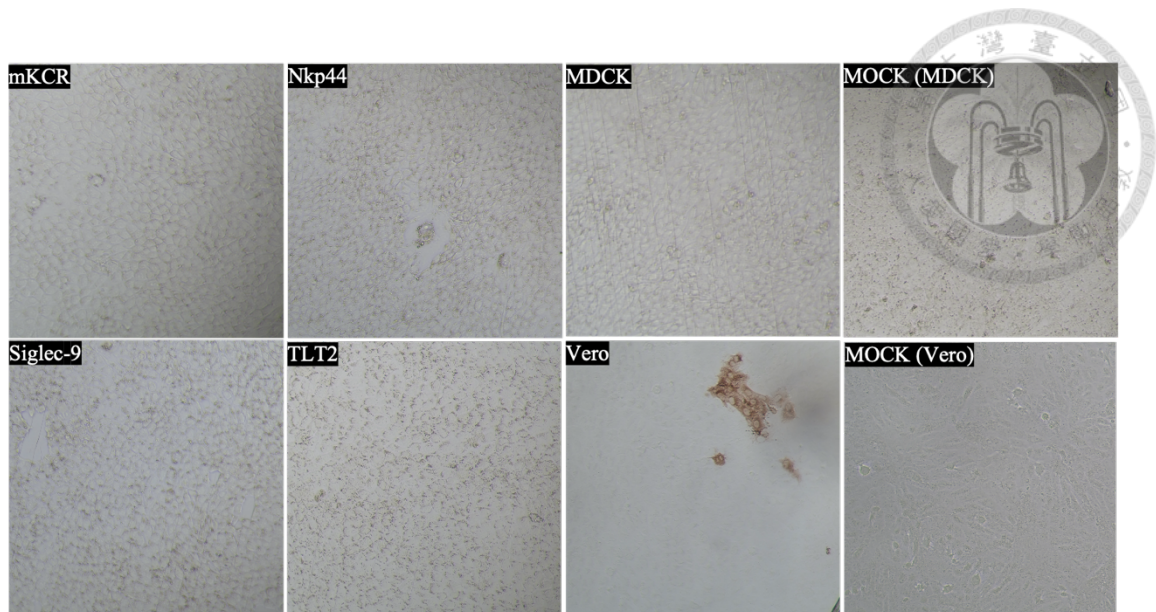


Fig. 4. Immunocytochemical staining in detection of PEDV infection using the anti-N protein antibody, PEDV inoculated in different lectin-encoded plasmids transfected MDCK cells. At 48 hours post infection with PEDV, MDCK cells were fixed with iced-cold 80% acetone and detected by the P4B antibody, the mouse anti-N protein antibody, and visualized by the EnVision-DAB+ system. The Vero cells were used as positive control.

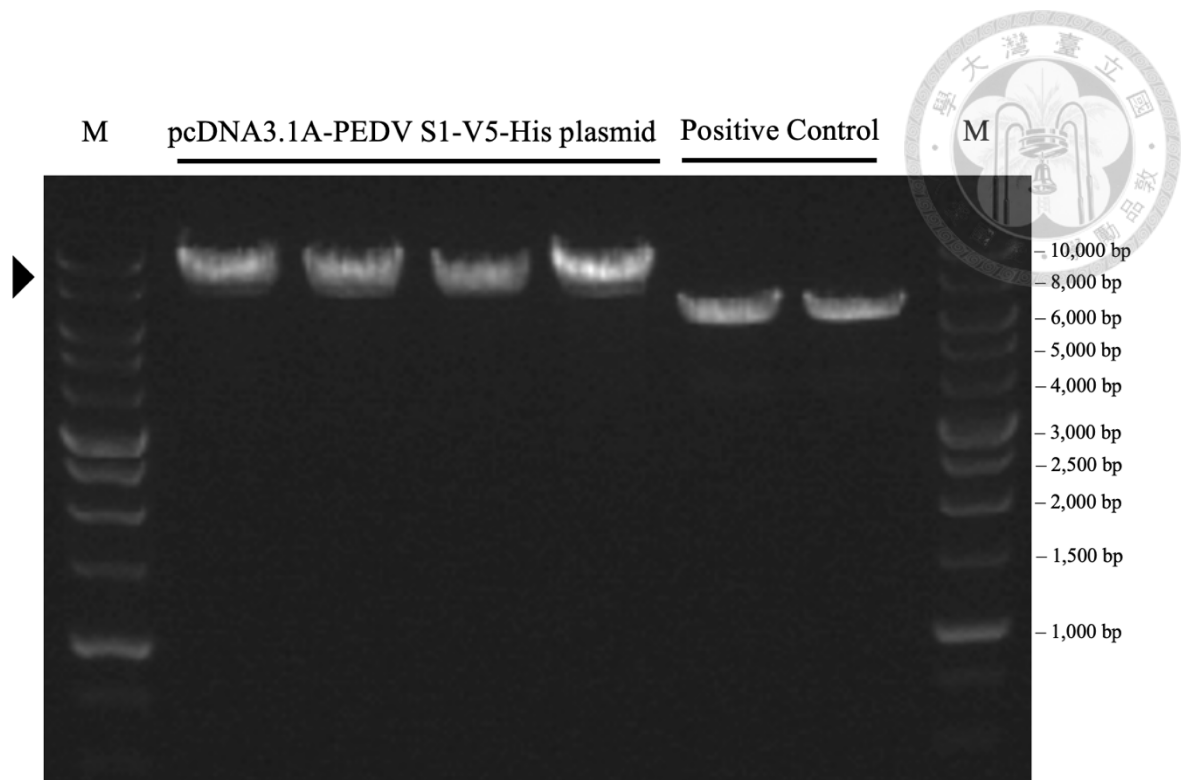
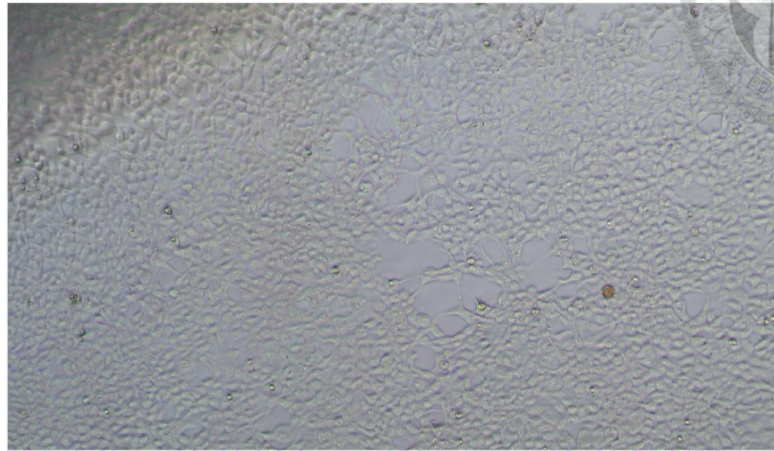


Fig. 5. Linearization of the pcDNA3.1-PEDV S1-V5-His plasmid (approximately 7.8 kb). The recombinant plasmid pcDNA3.1-PEDV S1-V5-His was successfully linearized using restriction enzyme digestion to generate a suitable DNA template for *in vitro* transcription. Concurrently, the laboratory-stock pcDNA3.1A/GP5/V5-His plasmid was processed in parallel to serve as the experimental positive control. Agarose gel electrophoresis confirmed a single, distinct DNA band of approximately 7.8 kb, corresponding to the expected size of the linearized construct, which was subsequently used for mRNA synthesis.

(A)



(B)

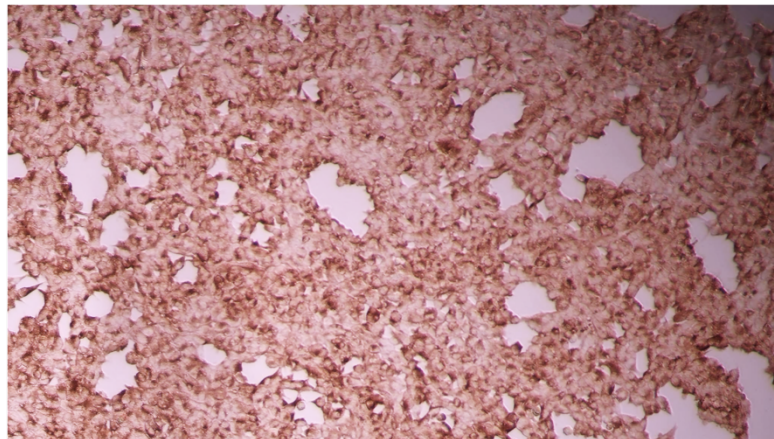


Fig. 6. The positive expression rate of PEDV S1 is enhanced after LNP encapsulation, as detected by ICC staining. ICC analysis was performed to compare S1 protein expression before and after LNP encapsulation. At 24 hours post transfection, cells were fixed with iced-cold 80% acetone, stained with the primary antibody, V5 tag Monoclonal antibody and detected by EnVision-DAB+ system. (A) Detection of PEDV S1 protein expression in HEK 293 cells transfected with naked PEDV S1 mRNA (B) Expression of PEDV S1 in HEK 293 cells transfected with LNP-encapsulated PEDV S1 mRNA

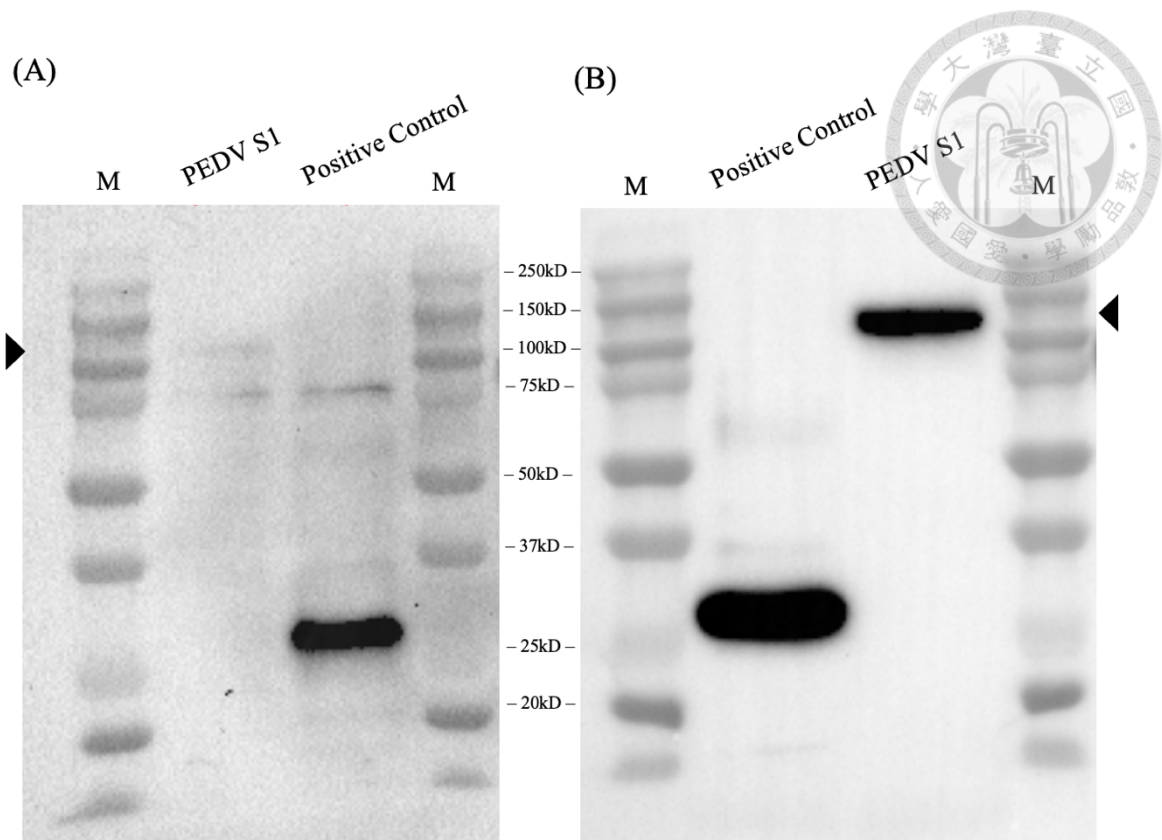


Fig. 7. Protein expression level of PEDV S1 (approx. 100 kDa) detected by western blot. The expression of PEDV S1 in HEK293 cells was verified at 24 hours post-transfection by western blot analysis, utilizing an anti-V5 tag mouse monoclonal antibody and visualized through chemiluminescence detection. mRNA derived from the pcDNA3.1A/GP5/V5-His plasmid was processed in parallel from the *in vitro* transcription phase and transfected to serve as the system-wide positive control, yielding a distinct protein band at approximately 25 kDa. (A) Detection of PEDV S1 protein expression in HEK 293 cells transfected with naked PEDV S1 mRNA (B) Expression of PEDV S1 in HEK 293 cells transfected with LNP-encapsulated PEDV S1 mRNA

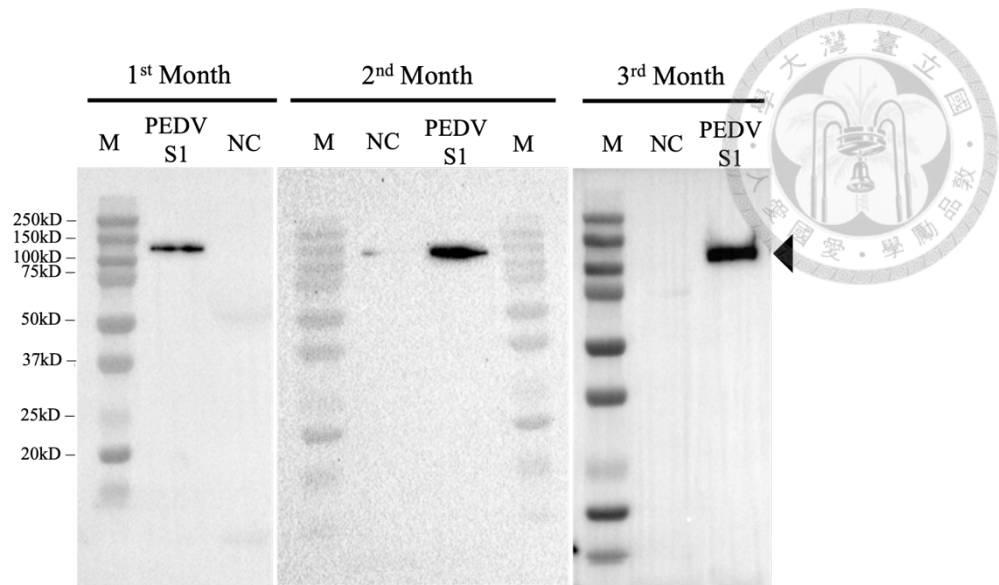


Fig. 8. The stability of LNP-encapsulated PEDV S1 mRNA over a three-month storage period. At 24 hours post transfection, the expression of PEDV S1 in one month-, two month-, and three month-storage period of LNP-formulated PEDV S1 mRNA transfected HEK 293 cells were detected by western blot analysis utilizing an anti-V5 tag mouse monoclonal antibody and visualized through chemiluminescence detection. (NC: Negative control)

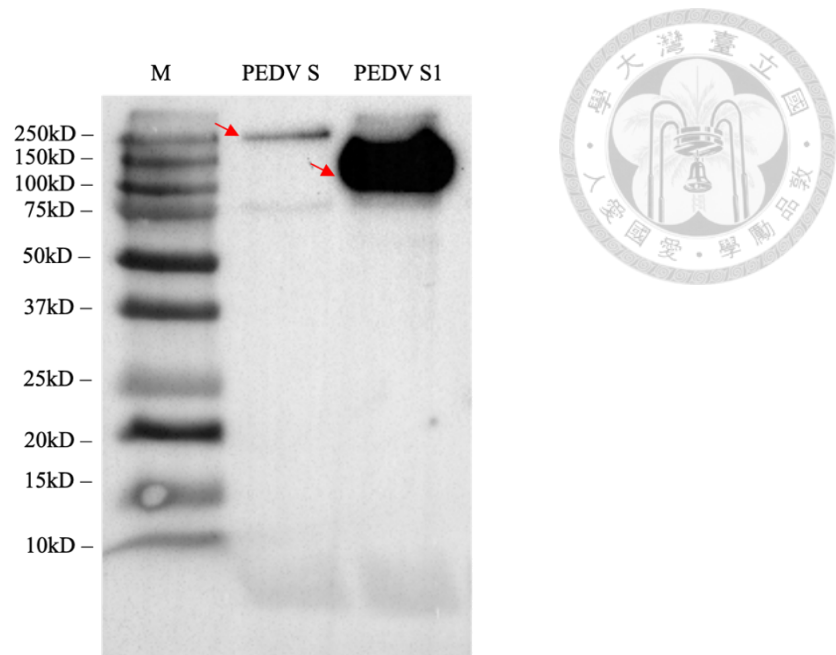


Fig. 9. Comparison of protein expression levels of full-length PEDV S and S1 subunit in HEK293 cell lysates collected at 48 hours of transient transfection with pcDNA3.1/PEDV S/V5-His plasmid and pcDNA3.1/PEDV S1/V5-His plasmids, respectively. The PEDV S protein and PEDV S1 subunit protein were detected by western blot analysis utilizing an anti-V5 tag mouse monoclonal antibody and visualized through chemiluminescence detection.

References



- Alameh, M.-G., Semon, A., Bayard, N. U., Pan, Y.-G., Dwivedi, G., Knox, J., Glover, R. C., Rangel, P. C., Tanes, C., & Bittinger, K. (2024). A multivalent mRNA-LNP vaccine protects against *Clostridioides difficile* infection. *Science*, 386(6717), 69–75.
- Alameh, M.-G., Tombácz, I., Bettini, E., Lederer, K., Ndeupen, S., Sittplangkoon, C., Wilmore, J. R., Gaudette, B. T., Soliman, O. Y., & Pine, M. (2021). Lipid nanoparticles enhance the efficacy of mRNA and protein subunit vaccines by inducing robust T follicular helper cell and humoral responses. *Immunity*, 54(12), 2877–2892. e2877.
- Annamalai, T., Saif, L. J., Lu, Z., & Jung, K. (2015). Age-dependent variation in innate immune responses to porcine epidemic diarrhea virus infection in suckling versus weaned pigs. *Veterinary immunology and immunopathology*, 168(3-4), 193–202.
- Aricescu, A. R., & Owens, R. J. (2013). Expression of recombinant glycoproteins in mammalian cells: towards an integrative approach to structural biology. *Current opinion in structural biology*, 23(3), 345–356.
- Boyd, W. C., & Shapleigh, E. (1954). Specific precipitating activity of plant agglutinins (lectins). *Science*, 119(3091), 419–419.

Brenner, S., Jacob, F., & Meselson, M. (1961). An unstable intermediate carrying information from genes to ribosomes for protein synthesis. *Nature*, 190(4776), 576–581.



Chang, C.-Y., Cheng, I.-C., Chang, Y.-C., Tsai, P.-S., Lai, S.-Y., Huang, Y.-L., Jeng, C.-R., Pang, V. F., & Chang, H.-W. (2019). Identification of neutralizing monoclonal antibodies targeting novel conformational epitopes of the porcine epidemic diarrhoea virus spike protein. *Scientific reports*, 9(1), 2529.

Chang, C.-Y., Hsu, W.-T., Chao, Y.-C., & Chang, H.-W. (2018). Display of porcine epidemic diarrhea virus spike protein on baculovirus to improve immunogenicity and protective efficacy. *Viruses*, 10(7), 346.

Chang, C.-Y., Wang, Y.-S., Wu, J.-F., Yang, T.-J., Chang, Y.-C., Chae, C., Chang, H.-W., & Hsu, S.-T. D. (2021). Generation and characterization of a spike glycoprotein domain A-specific neutralizing single-chain variable fragment against porcine epidemic diarrhea virus. *Vaccines*, 9(8), 833.

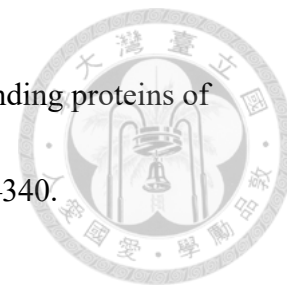
Chang, S.-H., Bae, J.-L., Kang, T.-J., Kim, J., Chung, G.-H., Lim, C.-W., Laude, H., Yang, M.-S., & Jang, Y.-S. (2002). Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. *Molecules and cells*, 14(2), 295–299.



- Chaudhary, N., Weissman, D., & Whitehead, K. A. (2021). mRNA vaccines for infectious diseases: principles, delivery and clinical translation. *Nature reviews Drug discovery*, 20(11), 817–838.
- Chen, S.-T., Lin, Y.-L., Huang, M.-T., Wu, M.-F., Cheng, S.-C., Lei, H.-Y., Lee, C.-K., Chiou, T.-W., Wong, C.-H., & Hsieh, S.-L. (2008). CLEC5A is critical for dengue-virus-induced lethal disease. *Nature*, 453(7195), 672–676.
- Chiou, H. Y., Huang, Y. L., Deng, M. C., Chang, C. Y., Jeng, C. R., Tsai, P. S., Yang, C., Pang, V., & Chang, H. W. (2017). Phylogenetic analysis of the spike (S) gene of the new variants of porcine epidemic diarrhoea virus in Taiwan. *Transboundary and Emerging Diseases*, 64(1), 157–166.
- Corbett, K. S., Edwards, D. K., Leist, S. R., Abiona, O. M., Boyoglu-Barnum, S., Gillespie, R. A., Himansu, S., Schäfer, A., Ziwawo, C. T., & DiPiazza, A. T. (2020). SARS-CoV-2 mRNA vaccine design enabled by prototype pathogen preparedness. *Nature*, 586(7830), 567–571.
- Crocker, P. R., Paulson, J. C., & Varki, A. (2007). Siglecs and their roles in the immune system. *Nature Reviews Immunology*, 7(4), 255–266.
- Cui, T., Theuns, S., Xie, J., Broeck, W. V. d., & Nauwynck, H. J. (2020). Role of porcine aminopeptidase N and sialic acids in porcine coronavirus infections in primary porcine enterocytes. *Viruses*, 12(4), 402.



- Cullis, P. R., & Hope, M. J. (2017). Lipid nanoparticle systems for enabling gene therapies. *Molecular Therapy*, 25(7), 1467–1475.
- Dam, T. K., & Brewer, C. F. (2010). Lectins as pattern recognition molecules: the effects of epitope density in innate immunity. *Glycobiology*, 20(3), 270–279.
- Deng, F., Ye, G., Liu, Q., Navid, M. T., Zhong, X., Li, Y., Wan, C., Xiao, S., He, Q., & Fu, Z. F. (2016). Identification and comparison of receptor binding characteristics of the spike protein of two porcine epidemic diarrhea virus strains. *Viruses*, 8(3), 55.
- Deng, X., van Geelen, A., Buckley, A. C., O'Brien, A., Pillatzki, A., Lager, K. M., Faaberg, K. S., & Baker, S. C. (2019). Coronavirus endoribonuclease activity in porcine epidemic diarrhea virus suppresses type I and type III interferon responses. *Journal of virology*, 93(8), 10.1128/jvi. 02000–02018.
- Drickamer, K. (1992). Engineering galactose-binding activity into a C-type mannose-binding protein. *Nature*, 360(6400), 183–186.
- Gabius, H.-J., André, S., Kaltner, H., & Siebert, H.-C. (2002). The sugar code: functional lectinomics. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1572(2-3), 165–177.
- Gerdt, V., & Zakhartchouk, A. (2017). Vaccines for porcine epidemic diarrhea virus and other swine coronaviruses. *Veterinary microbiology*, 206, 45–51.



Goldstein, I. J., & Hayes, C. E. (1978). The lectins: carbohydrate-binding proteins of plants and animals. *Adv Carbohydr Chem Biochem*, 35, 127–340.

[https://doi.org/10.1016/s0065-2318\(08\)60220-6](https://doi.org/10.1016/s0065-2318(08)60220-6)

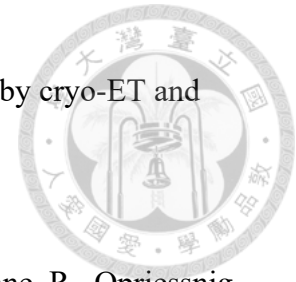
Gustafsson, C., Govindarajan, S., & Minshull, J. (2004). Codon bias and heterologous protein expression. *Trends in biotechnology*, 22(7), 346–353.

Hershkovitz, O., Rosental, B., Rosenberg, L. A., Navarro-Sanchez, M. E., Jivov, S., Zilka, A., Gershoni-Yahalom, O., Brient-Litzler, E., Bedouelle, H., & Ho, J. W. (2009). NKp44 receptor mediates interaction of the envelope glycoproteins from the West Nile and dengue viruses with NK cells. *The Journal of Immunology*, 183(4), 2610–2621.

Ho, J. W., Hershkovitz, O., Peiris, M., Zilka, A., Bar-Ilan, A., Nal, B., Chu, K., Kudelko, M., Kam, Y. W., & Achdout, H. (2008). H5-type influenza virus hemagglutinin is functionally recognized by the natural killer-activating receptor NKp44. *Journal of virology*, 82(4), 2028–2032.

Hou, X., Zaks, T., Langer, R., & Dong, Y. (2021). Lipid nanoparticles for mRNA delivery. *Nature Reviews Materials*, 6(12), 1078–1094.

Huang, C.-Y., Draczkowski, P., Wang, Y.-S., Chang, C.-Y., Chien, Y.-C., Cheng, Y.-H., Wu, Y.-M., Wang, C.-H., Chang, Y.-C., & Chang, Y.-C. (2022). In situ



structure and dynamics of an alphacoronavirus spike protein by cryo-ET and cryo-EM. *Nature communications*, 13(1), 4877.

Huang, Y.-W., Dickerman, A. W., Piñeyro, P., Li, L., Fang, L., Kiehne, R., Opriessnig,

T., & Meng, X.-J. (2013). Origin, evolution, and genotyping of emergent

porcine epidemic diarrhea virus strains in the United States. *MBio*, 4(5),

10.1128/mbio.00737–00713.

Ji, C. M., Wang, B., Zhou, J., & Huang, Y. W. (2018). Aminopeptidase-N-independent

entry of porcine epidemic diarrhea virus into Vero or porcine small intestine

epithelial cells. *Virology*, 517, 16–23. <https://doi.org/10.1016/j.virol.2018.02.019>

Jung, K., & Saif, L. J. (2015). Porcine epidemic diarrhea virus infection: Etiology,

epidemiology, pathogenesis and immunoprophylaxis. *The Veterinary Journal*,

204(2), 134–143.

Jung, K., Saif, L. J., & Wang, Q. (2020). Porcine epidemic diarrhea virus (PEDV): An

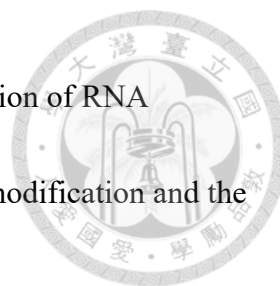
update on etiology, transmission, pathogenesis, and prevention and control.

Virus Res, 286, 198045. <https://doi.org/10.1016/j.virusres.2020.198045>

Jung, K., Wang, Q., Scheuer, K. A., Lu, Z., Zhang, Y., & Saif, L. J. (2014). Pathology

of US porcine epidemic diarrhea virus strain PC21A in gnotobiotic pigs.

Emerging infectious diseases, 20(4), 662.



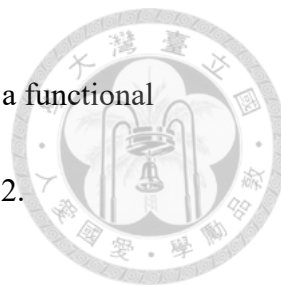
Karikó, K., Buckstein, M., Ni, H., & Weissman, D. (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity*, 23(2), 165–175.

Karikó, K., Muramatsu, H., Welsh, F. A., Ludwig, J., Kato, H., Akira, S., & Weissman, D. (2008). Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Molecular Therapy*, 16(11), 1833–1840.

Kowalski, P. S., Rudra, A., Miao, L., & Anderson, D. G. (2019). Delivering the messenger: advances in technologies for therapeutic mRNA delivery. *Molecular Therapy*, 27(4), 710–728.

Langel, S. N., Paim, F. C., Lager, K. M., Vlasova, A. N., & Saif, L. J. (2016). Lactogenic immunity and vaccines for porcine epidemic diarrhea virus (PEDV): Historical and current concepts. *Virus Res*, 226, 93–107.
<https://doi.org/10.1016/j.virusres.2016.05.016>

Langel, S. N., Wang, Q., Vlasova, A. N., & Saif, L. J. (2020). Host factors affecting generation of immunity against porcine epidemic diarrhea virus in pregnant and lactating swine and passive protection of neonates. *Pathogens*, 9(2), 130.



- Li, B. X., Ge, J. W., & Li, Y. J. (2007). Porcine aminopeptidase N is a functional receptor for the PEDV coronavirus. *Virology*, 365(1), 166–172.
<https://doi.org/10.1016/j.virol.2007.03.031>
- Li, W., Luo, R., He, Q., van Kuppeveld, F. J. M., Rottier, P. J. M., & Bosch, B. J. (2017). Aminopeptidase N is not required for porcine epidemic diarrhea virus cell entry. *Virus Res*, 235, 6–13. <https://doi.org/10.1016/j.virusres.2017.03.018>
- Li, W., van Kuppeveld, F. J., He, Q., Rottier, P. J., & Bosch, B.-J. (2016). Cellular entry of the porcine epidemic diarrhea virus. *Virus research*, 226, 117–127.
- Li., Van Kuppeveld, F. J. M., He, Q., Rottier, P. J. M., & Bosch, B. J. (2016). Cellular entry of the porcine epidemic diarrhea virus. *Virus Res*, 226, 117–127.
<https://doi.org/10.1016/j.virusres.2016.05.031>
- Lin, C. M., Saif, L. J., Marthaler, D., & Wang, Q. (2016). Evolution, antigenicity and pathogenicity of global porcine epidemic diarrhea virus strains. *Virus Res*, 226, 20–39. <https://doi.org/10.1016/j.virusres.2016.05.023>
- Lin, C. N., Chung, W. B., Chang, S. W., Wen, C. C., Liu, H., Chien, C. H., & Chiou, M. T. (2014). US-like strain of porcine epidemic diarrhea virus outbreaks in Taiwan, 2013-2014. *J Vet Med Sci*, 76(9), 1297–1299.
<https://doi.org/10.1292/jvms.14-0098>

Makadiya, N., Brownlie, R., van den Hurk, J., Berube, N., Allan, B., Gerdts, V., &

Zakhartchouk, A. (2016). S1 domain of the porcine epidemic diarrhea virus spike protein as a vaccine antigen. *Virology journal*, 13(1), 57.

Mauro, V. P., & Chappell, S. A. (2014). A critical analysis of codon optimization in human therapeutics. *Trends in molecular medicine*, 20(11), 604–613.

Mohamadzadeh, M., Coberley, S. S., Olinger, G. G., Kalina, W. V., Ruthel, G., Fuller, C. L., Swenson, D. L., Pratt, W. D., Kuhns, D. B., & Schmaljohn, A. L. (2006). Activation of triggering receptor expressed on myeloid cells-1 on human neutrophils by marburg and ebola viruses. *Journal of virology*, 80(14), 7235–7244.

Nam, E., & Lee, C. (2010). Contribution of the porcine aminopeptidase N (CD13) receptor density to porcine epidemic diarrhea virus infection. *Vet Microbiol*, 144(1-2), 41–50. <https://doi.org/10.1016/j.vetmic.2009.12.024>

Oh, J., Lee, K.-W., Choi, H.-W., & Lee, C. (2014). Immunogenicity and protective efficacy of recombinant S1 domain of the porcine epidemic diarrhea virus spike protein. *Archives of virology*, 159(11), 2977–2987.

Osorio, F., & e Sousa, C. R. (2011). Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity*, 34(5), 651–664.

Pardi, N., Hogan, M. J., Naradikian, M. S., Parkhouse, K., Cain, D. W., Jones, L.,

Moody, M. A., Verkerke, H. P., Myles, A., & Willis, E. (2018a). Nucleoside-modified mRNA vaccines induce potent T follicular helper and germinal center B cell responses. *Journal of Experimental Medicine*, 215(6), 1571–1588.

Pardi, N., Hogan, M. J., Porter, F. W., & Weissman, D. (2018b). mRNA vaccines—a new era in vaccinology. *Nature reviews Drug discovery*, 17(4), 261–279.

Pensaert, M., & De Bouck, P. (1978). A new coronavirus-like particle associated with diarrhea in swine. *Archives of virology*, 58(3), 243–247.

Sahin, U., Karikó, K., & Türeci, Ö. (2014). mRNA-based therapeutics—developing a new class of drugs. *Nature reviews Drug discovery*, 13(10), 759–780.

Schoenmaker, L., Witzigmann, D., Kulkarni, J. A., Verbeke, R., Kersten, G., Jiskoot, W., & Crommelin, D. J. (2021). mRNA-lipid nanoparticle COVID-19 vaccines: Structure and stability. *International journal of pharmaceutics*, 601, 120586.

Sharon, N., & Lis, H. (2004). History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiology*, 14(11), 53R–62R.

Song, D., & Park, B. (2012). Porcine epidemic diarrhoea virus: a comprehensive review of molecular epidemiology, diagnosis, and vaccines. *Virus genes*, 44(2), 167–175.

Stevenson, G. W., Hoang, H., Schwartz, K. J., Burrough, E. R., Sun, D., Madson, D.,

Cooper, V. L., Pillatzki, A., Gauger, P., Schmitt, B. J., Koster, L. G., Killian, M.

L., & Yoon, K. J. (2013). Emergence of Porcine epidemic diarrhea virus in the

United States: clinical signs, lesions, and viral genomic sequences. *J Vet Diagn*

Invest, 25(5), 649–654. <https://doi.org/10.1177/1040638713501675>

Subramaniam, S., Cao, D., Tian, D., Cao, Q. M., Overend, C., Yugo, D. M., Matzinger,

S. R., Rogers, A. J., Heffron, C. L., & Catanzaro, N. (2017). Efficient priming of

CD4 T cells by Langerin-expressing dendritic cells targeted with porcine

epidemic diarrhea virus spike protein domains in pigs. *Virus research*, 227, 212–

219.

Teijaro, J. R., & Farber, D. L. (2021). COVID-19 vaccines: modes of immune

activation and future challenges. *Nature Reviews Immunology*, 21(4), 195–197.

Tortorici, M. A., & Vesler, D. (2019). Structural insights into coronavirus entry. In

Advances in virus research (Vol. 105, pp. 93–116). Elsevier.

Trepotec, Z., Geiger, J., Plank, C., Aneja, M. K., & Rudolph, C. (2019). Segmented

poly (A) tails significantly reduce recombination of plasmid DNA without

affecting mRNA translation efficiency or half-life. *Rna*, 25(4), 507–518.

Tsai, Y.-X., Chang, N.-E., Reuter, K., Chang, H.-T., Yang, T.-J., von Bülow, S.,

Sehrawat, V., Zerrouki, N., Tuffery, M., & Gecht, M. (2024). Rapid simulation



- of glycoprotein structures by grafting and steric exclusion of glycan conformer libraries. *Cell*, 187(5), 1296–1311. e1226.
- Van Breedam, W., Pohlmann, S., Favoreel, H. W., de Groot, R. J., & Nauwynck, H. J. (2014). Bitter-sweet symphony: glycan-lectin interactions in virus biology. *FEMS Microbiol Rev*, 38(4), 598–632. <https://doi.org/10.1111/1574-6976.12052>
- Wang, X., Chen, J., Shi, D., Shi, H., Zhang, X., Yuan, J., Jiang, S., & Feng, L. (2016). Immunogenicity and antigenic relationships among spike proteins of porcine epidemic diarrhea virus subtypes G1 and G2. *Archives of virology*, 161(3), 537–547.
- Watanabe, Y., Allen, J. D., Wrapp, D., McLellan, J. S., & Crispin, M. (2020). Site-specific glycan analysis of the SARS-CoV-2 spike. *Science*, 369(6501), 330–333.
- Weis, W. I., & Drickamer, K. (1996). Structural basis of lectin-carbohydrate recognition. *Annual review of biochemistry*, 65(1), 441–473.
- Wood, E. (1977). An apparently new syndrome of porcine epidemic diarrhoea.
- Zelensky, A. N., & Gready, J. E. (2005). The C-type lectin-like domain superfamily. *The FEBS journal*, 272(24), 6179–6217.

Zhang, Q., Ke, H., Blikslager, A., Fujita, T., & Yoo, D. (2018). Type III interferon restriction by porcine epidemic diarrhea virus and the role of viral protein nsp1 in IRF1 signaling. *Journal of virology*, 92(4), 10.1128/jvi. 01677–01617.

Zhang, Y., Chen, Y., Zhou, J., Wang, X., Ma, L., Li, J., Yang, L., Yuan, H., Pang, D., & Ouyang, H. (2022). Porcine epidemic diarrhea virus: an updated overview of virus epidemiology, virulence variation patterns and virus–host interactions. *Viruses*, 14(11), 2434.

Zhao, P., Xu, L.-D., Zhang, Y., Cao, H., Chen, R., Wang, B., & Huang, Y.-W. (2021). Expression of the human or porcine C-type lectins DC-SIGN/L-SIGN confers susceptibility to porcine epidemic diarrhea virus entry and infection in otherwise refractory cell lines. *Microbial Pathogenesis*, 157, 104956.

Zhao, Y., Fan, B., Song, X., Gao, J., Guo, R., Yi, C., He, Z., Hu, H., Jiang, J., & Zhao, L. (2024). PEDV-spike-protein-expressing mRNA vaccine protects piglets against PEDV challenge. *MBio*, 15(2), e02958–02923.

Zou, Z., Chastain, A., Moir, S., Ford, J., Trandem, K., Martinelli, E., Cicala, C., Crocker, P., Arthos, J., & Sun, P. D. (2011). Siglecs facilitate HIV-1 infection of macrophages through adhesion with viral sialic acids. *PloS one*, 6(9), e24559.