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Research Article

Safety Evaluation of Recombinant Bovine Lactoferrin as a Novel Biomaterial

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Abstract

This study introduces the physical principles and safety evaluation of recombinant bovine lactoferrin (fusion factor) as an innovative biomaterial. Fusion factor is a recombinant lactoferrin expressed by fusing lactoferrin, which has natural biological defense function, with other peptide segments through sequence optimization. It is named fusion factor. Its molecular weight is about 36kDa, which is much greater than the 1kDa molecular weight limit of macromolecular transdermal absorption, so it is not absorbed when used externally on the epithelial mucosa. The lactoferrin based biological defense functional peptide segment in the fusion factor can neutralize the virus by binding to viral protein nucleic acid through the physical action of charge adsorption, and can also compete with cell receptors to inhibit virus infection in cells. The molar ratio of the transmembrane peptide (Pep-1) fragment to the carrier protein is 1:1, so only the transport protein is anchored to the cell surface, forming a physical isolation protein protective wall against viruses and bacteria, without penetrating the cell or damaging the cell membrane. The fusion factor and its derived vaginal bacteria blocking gel have no significant toxicity, sensitization, anaphylaxis or delayed hypersensitivity in vitro cell experiments, in vivo animal experiments and clinical observation tests, and have no side effects with highly safety.

Keywords

Recombinant Bovine Lactoferrin, Fusion Factor, Biological Defense, Physical Effects, Security

1. Introduction

Natural biological defense factors (defensins) are distributed in almost all biological groups [1], mainly through physical binding with virus coat proteins, causing virus inactivation, making it difficult for microorganisms to develop resistance [2]. Most defense factors can be developed and produced through genetic engineering and protein recombination technology [3]. There are many naturally occurring proteins with biological defense functions in mammalian milk, which form part of the defense mechanism mediated by the mother to the offspring through milk [4].

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Lactoferrin (LF, Uniprot P24627, NM_180998.2) is an iron bound single chain peptide glycoprotein consisting of approximately 703 amino acids and a molecular weight of approximately 80kDa. It is mainly secreted by glandular cells and distributed on the surface of mucosal epithelial cells and body fluids. Natural secretion is found in human and mammalian milk, intestines, vagina, and oral tissues. Lactoferrin and its derived peptides are not only the main nutritional components in milk, but also one of the natural biological defense factors. However, their pharmacological concentration is much higher than the actual physiological concentration, and animal experiments have no effect, indicating that lactoferrin cannot be directly absorbed and has a high concentration of action alone. Under physiological conditions, lactoferrin needs to work in synergy with other biological defense proteins [5, 6]. Lactoferrin, due to its high safety, has been mainly used as a dietary supplement or nutritional supplement for dairy products since its discovery in 1939. The separation of natural lactoferrin and its functional peptide segments is costly, inefficient, and the process is complex. Expression of lactoferrin through heterologous recombination can reduce costs and improve efficiency, but absorption and efficacy are still poor [7].

Cell Penetrating Peptides (CPP) bind to the carrier material, insert into the phospholipid bilayer of the membrane structure, change the surface charge distribution and physical binding between the carrier material and the membrane structure, and induce spatial conformational changes in the membrane structure [8]. The transmembrane penetration ability of PEP-1 is unique, as its molar ratio to the carrier material increases, transitioning from anchoring the cell membrane to penetrating the cell membrane [9]. The Chinese Academy of Biomedical Sciences, in collaboration with scientists from renowned research institutions such as South China University of Technology, Wuhan Institute of Virology of the Chinese Academy of Sciences, and the American Academy of Nanomedicine, creatively utilized the cell membrane anchoring function of the special transmembrane penetrating peptide PEP-1 based on the biological defense functional peptide segment of lactoferrin, added other peptide segments for fusion expression, optimized expression conditions using purified tag peptide segments, and increased expression levels. Finally, the optimized and modified recombinant lactoferrin is named fusion factor and registered as PHPV. Based on this new biomaterial, this study selects the vaginal external gel dosage form, develops the PHPV fusion factor vaginal bacteria blocking gel, and determines the safety of the fusion factor and its derivative medical devices through cell models, animal vaginal models and clinical observation tests.

2. Materials and Methods

2.1. Cytotoxicity Testing

Wuhan Institute of Virology, Chinese Academy of Sciences was entrusted to use HEK293 cell culture model and MTS kit to detect the potential cytotoxicity of fusion factor and its gel at different concentrations.

2.2. Pharmacological Activity Testing

Entrust the Guangzhou Institute of Microbiology to apply the "Disinfection Technical Specifications" (2002 Edition) 2.1.8.2 Antibacterial Loop Test of the Ministry of Health to evaluate whether the 1mg/ml fusion factor and standard reference lactoferrin (Sigma, L4765-50MG) have reached pharmacological inhibitory concentrations against Staphylococcus aureus (ATCC 6538) and Lactobacillus acidophilus (CICC 6005).

2.3. Guinea Pig Skin Irritation Detection

Entrust Guangdong Newei Quality and Technology Laboratory to conduct guinea pig experiments in accordance with the recommended delayed type hypersensitivity test method in the national standard GB/T16886.10-2005 "Biological Evaluation of Medical Devices Part 10: Stimulation and Delayed Type Hypersensitivity Test". Grade the skin reactions at the stimulation site of the sample and control group animals, while observing the weight, growth, and clinical gross changes of the animals.

2.4. Vaginal Mucosal Irritation Detection

Entrust Guangdong Newei Quality and Technology Laboratory to conduct vaginal irritation tests according to the recommended method in Appendix B of GB/T16886.10-2005 "Biological Evaluation of Medical Devices Part 10: Stimulation and Delayed Type Hypersensitivity Test". Check for irritation, damage, and necrosis of the epithelial tissue layer. After fixation with 4% formaldehyde solution in vaginal tissue, histological evaluation of pathological sections was performed.

2.5. Vaginal Mucosal Permeability Testing

Entrust Boxi General Testing Laboratory to conduct vaginal irritation testing according to the recommended method in Appendix B of GB/T16886.10-2005 "Biological Evaluation of Medical Devices Part 10: Stimulation and Delayed Type Hypersensitivity Test". The FITC labeled fusion factor gel was applied to the vaginal mucosa of rabbits, and the animals were killed at different time points. Fluorescence microscopy was used to observe the fluorescence of frozen sections, and pathological HE staining was used to determine the tissue location.

2.6. Statistical Data Analysis

All data are presented as mean±standard deviation, unless stated otherwise. Statistical analyses were performed by the

t-test; $p<0.05$ was considered statistically significant.

3. Result

3.1. Fusion Factor Has No Cytotoxicity

As shown in Figure 1, the lowest to highest concentration of fusion factor detected is about 1mg/ml, which has no cytotoxicity to HEK293 cells, and its gel formula has no significant cell growth inhibition.

3.2. Pharmacological Activity Testing

As shown in Table 1, Sigma lactoferrin standard and fusion factor showed no antibacterial effect on Staphylococcus aureus and Lactobacillus acidophilus at a test concentration of 1mg/ml, and did not reach the pharmacological concentration.

3.3. Fusion Factor Gel Has No Animal Toxicity and Skin Irritation

As shown in Table 2, the weight, growth and clinical observation of animals in the experimental group (fusion factor gel) and the negative control group showed no significant changes, and the positive incidence of sensitization reaction in guinea pigs was 0% (Table 3).

3.4. Fusion Factor Gel Has No Irritation of Vaginal Mucosa

As shown in Table 4 and Table 5, compared with the control group, the sample group (fusion factor gel) has no abnormalities in the observation and anatomy of the rabbit vaginal vulva and vagina, and the irritation index recorded by the mucosal tissue reaction microscope is 2.55.

Table 1. *Results of fusion factor inhibitory concentration detection.*

number of samples	mutant test strain number	Bacterial suspension concentration (CFU/ml)	Diameter of antibacterial ring of each sample				
			1	2	3	4	5
	1	1.47×106	5.00	5.00	5.01	5.00	5.00
	2	1.52×106	5.00	5.00	5.01	5.01	5.01
XJ20231919-1	3	1.52×10^{6}	5.01	5.00	5.02	5.01	5.01
fusion factor	1	1.80×10^{6}	5.00	5.03	5.01	5.02	5.02
	2	1.79×106	5.03	5.00	5.00	5.01	5.01
	3	1.84×10^{6}	5.01	5.00	5.00	5.01	5.01
	1	1.47×106	5.00	5.02	5.00	5.03	5.01
	2	1.52×10^{6}	5.01	5.00	5.02	5.01	5.01
XJ20231920-1 sigma lactoferrin	3	1.52×106	5.00	5.00	5.01	5.03	5.01
standard	1	1.80×106	5.00	5.00	5.01	5.00	5.00
	2	1.79×106	5.00	5.01	5.02	5.02	5.01
	3	1.84×106	5.01	5.01	5.00	5.00	5.01

Note:

1) The diameter of the negative control antibacterial ring is 5mm.

2) According to 2.1.8.2 of the "Disinfection Technical Specification" (2002 edition), if the diameter of the antibacterial ring is greater than 7mm, it is judged to have antibacterial effect; If the diameter of the antibacterial ring is less than or equal to 7mm, it is judged as having no antibacterial effect.

Table 2. *Clinical observation and weight changes of guinea pigs.*

grouping	animal number	Weight (g)		Clinical manifestations other than skin reactions
		Initial weight	Final weight	
experimenta group	1	349	387	normal
	2	324	413	normal
	3	348	463	normal
	4	349	370	normal
	5	375	459	normal
	6	362	432	normal
	7	378	399	normal
	8	341	429	normal
	9	351	382	normal
	10	370	389	normal
Negative control group	1	331	390	normal
	2	425	510	normal
	3	420	485	normal
	4	330	398	normal
	5	370	456	normal

Table 3. *Sensitized skin reactions of guinea pigs.*

grouping	Animal number	time after the excitation application was removed		Positive probability after excitation
		24h	48h	
Negative control group	1	0	0	0%
	2	0	0	
	3	0	0	
	4	0	0	
	5	0	0	
	6	0	0	
	7	0	0	
	8	0	0	
	9	0	0	
	10	0	0	
	1	0	0	0%
	2	0	0	
	3	0	0	
	4	0	0	
	5	0	0	

Table 4. Vaginal (perineum) observation and vaginal anatomy table.

group	rabbit number	Vaginal observation		Vaginal anatomy	
		no abnormality seen	Abnormal condition	no abnormality Seen	Abnormal condition
Sample group	1#	√	/	√	/
	2#	√	/	√	/
	3#	√	/	√	/
Control group	1#	√	/	√	/
	2#		/	√	/
	3#	√	/	√	/

Table 5. Results of vaginal tissue response microscope score.

group	rabbit number	tissue response microscopy score												separating score	average score	stimulation index
		epithelium			Leukocytic infiltration			congestion of blood vessel			edema					
sample group	1#	2	1	1	1	1	1	0	1	1	0	1	3	4.33	4.66	2.55
	2#	0	0	1	1	1	1	0	0	1	1	2	1	3.00		
	3#	3	3	3	2	3	3	0	0	1	1	0	1	6.67		
control group	1#	0	0	0	0	0	0	0	0	0	2	2	2	2.00	2.11	
	2#	0	0	0	0	0	0	0	0	0	2	2	1	1.67		
	3#	1	1	1	0	0	0	1	0	2	1	1	1	2.67		

3.5. Fusion Factor Gel Vaginal Mucosa Is Impermeable

As shown in the Figure 2, FITC fluorescence labeled fusion factor (Figure 2A), after vaginal application of gel, was observed at different time points in comparison with pathological staining, and no mucosal infiltration or absorption fluorescence signal was detected (Figure 2B).

Thin layer chromatography identification of fusion factors labeled with fluorescent dye FITC; B Rabbit vagina was smeared with fluorescent staining of PHPV fusion factor vaginal bacteria blocking gel, and pathological HE staining was compared with fluorescence microscope at different time points.

Thin layer chromatography identification of fusion factors labeled with fluorescent dye FITC; B Rabbit vagina was smeared with fluorescent staining of PHPV fusion factor vaginal bacteria blocking gel, and pathological HE staining was compared with fluorescence microscope at different time points.

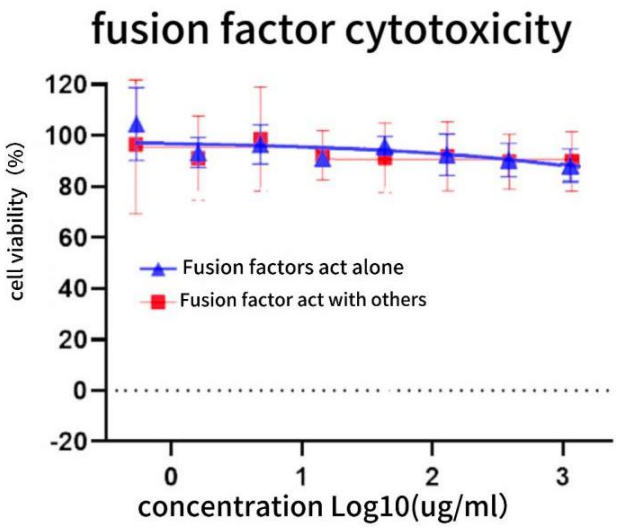

Figure 1. Fusion factor cytotoxicity detection.

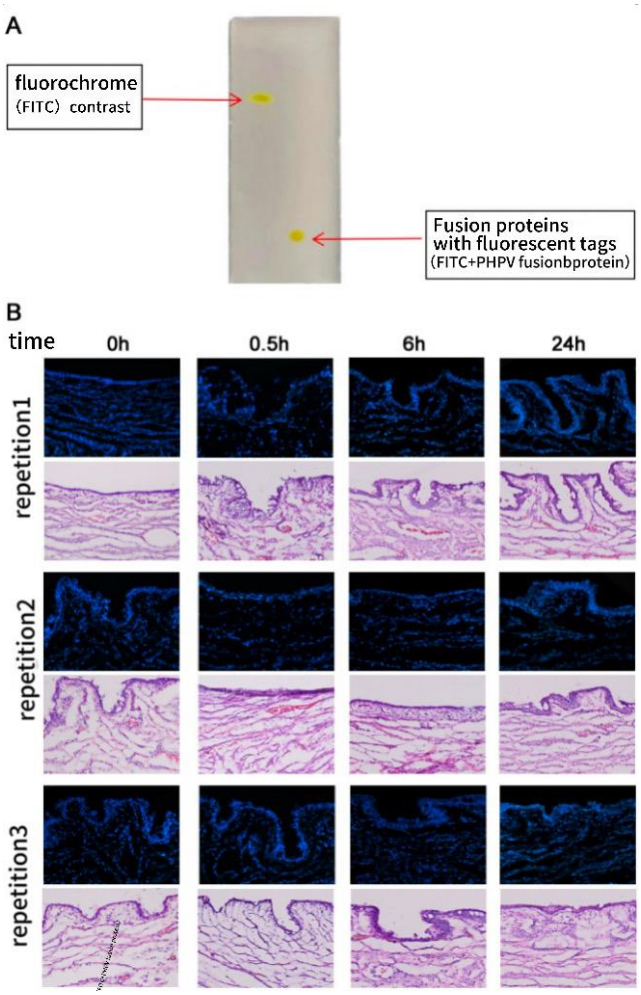

Figure 2. *Fusion factor gel vaginal mucosa penetration test results.*

4. Discussion

In order to combat HPV infection, scientists from the School of Biological Science and Engineering at South China University of Technology, the Institute of Virology at the Chinese Academy of Sciences, the Chinese Academy of Biomedical Sciences, and the American Academy of Nanomedicine have screened a biological defense factor that can block HPV infection from bovine lactoferrin, but it is naturally expressed less. In order to improve protein expression and enhance its antiviral function, the research and development team used genetic engineering technology to synthesize several peptide substances, Fusion expression into a novel biological defense function fusion protein with stronger antibacterial and antiviral functions, named: fusion factor, registered as: PHPV; R&D code: FLM-1. Its molecular weight of 36kDa is much greater than the limit of 1kDa for skin absorbable macromolecules in topical preparations, so it is not directly absorbed by the epidermis and does not produce pharmacological effects in epidermal applications. Reference data and clinical trial data indicate that lactoferrin is highly safe as a food [10], and recombinant lactoferrin is also very safe [11], but the concentration at which it acts alone also needs to be very high [12]. The fusion factor, also known as recombinant lactoferrin, inherits the high safety of lactoferrin on the one hand, and on the other hand, through unique innovative design, its Pep-1 peptide segment only plays a role in physically binding to the cell surface and virus surface for carrying biological defense functions, equivalent to anchoring to the cell membrane or virus surface, forming an antiviral protective wall [9, 13].

Through the change of spatial conformation, the fusion factor makes the surface charge of the antiviral functional peptide physically adsorb the positive and negative charge parts of the protein or nucleic acid that bind the virus, envelop the virus and spatially block the virus from binding to the cell membrane [14], or binding to its receptor [15], supplemented by the physical isolation membrane function of viscous gel, jointly inhibit the virus adsorption process through the physical mechanism, inactivate the virus infection ability, and play a role in preventing cervical lesions. In recent years, well-known academic research institutions in countries such as Harvard University, Yale University, NIH, the UK, Japan, Germany, and Spain have successively published experimental data on antiviral and antibacterial functional peptide segments designed with fusion factors, elucidating new physical mechanisms of antiviral action in different structural regions of fusion proteins, which has attracted widespread attention internationally [16-21].

Third party data such as the Institute of Virology of the Chinese Academy of Sciences also confirmed that the fusion factor and its derived vaginal bacteria blocking gel were not absorbed by the mucosa; Low concentrations do not exert pharmacological effects, but only have a physical effect of blocking viral infection in vitro. The fusion factor based on recombinant lactoferrin not only has high self safety, but also its derived vaginal bacteria blocking gel has no significant toxicity, sensitization, no allergy or delayed hypersensitivity, no side effects and high safety in vitro cell experiments and in vivo animal experiments. Therefore, fusion factors developed based on recombinant lactoferrin are expected to become new materials for antiviral and antibacterial biological defense, and will be more widely applied in the field of biomedicine.

5. Conclusion

This study introduces the physical principles and safety evaluation of recombinant bovine lactoferrin (fusion factor) as an innovative biomaterial. Fusion factor is a recombinant lactoferrin expressed by fusing lactoferrin, which has natural biological defense function, with other peptide segments through sequence optimization. The fusion factor and its derived vaginal bacteria blocking gel have no significant toxicity, sensitization, anaphylaxis or delayed hypersensitivity in vitro cell experiments, in vivo animal experiments and clinical observation tests, and have no side effects with highly safety.

6. Recommendations

This study confirms that the fusion factor and its derived vaginal antibacterial gel have good safety in both in vitro cell experiments and in vivo animal experiments. Therefore, the preventive and therapeutic effects of fusion factors on viral infections can be confirmed through further in vivo experiments and clinical trials.

Abbreviations

CPP: Cell Penetrating Peptides
PEP-1: Penetrating Peptide
PHPV: Peptide anti Human Papillomavirus
HPV: Human Papillomavirus
NIH: National Institutes of Health
UK: United Kingdom

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Author Contributions

Conceptualization, J. S.; S. Z. and Y. W.; data curation, J. S.; S. Z.; Y. T.; B. Q.; X. P.; and H. D.; writing—original draft preparation, J. S. and S. Z.; writing—review and editing, Y. W., J. W. and C. W. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

References

[1] Chen Y, Ge YQ, Zhang LM, et al. Research progress and application prospects of mammalian defense peptides. Progress in Biochemistry and Biophysics, 2001 https://doi.org/10.3321/j.issn: 1000-3282.2001.01.004

[2] Drayton M, Deisinger JP, Ludwig KC, Raheem N, Müller A, Schneider T, Straus SK. Host Defense Peptides: Dual Antimicrobial and Immunomodulatory Action. Int J Mol Sci. 2021 Oct 16; 22(20): 11172. https://doi.org/10.3390/ijms222011172

[3] Roca-Pinilla R, Lisowski L, Arís A, Garcia-Fruitós E. The future of recombinant host defense peptides. Microb Cell Fact. 2022 Dec 21; 21(1): 267. https://doi.org/10.1186/s12934-022-01991-2

[4] Ma XM, Su JX, Chen Y, et al. Research progress on the structure and function of four main proteins in animal milk and their separation and purification methods. Journal of Northwest University for Nationalities (Natural Science Edition), 2022, 43(02): 74-79. https://doi.org/10.14084/j.cnki.cn62-1188/n.2022.02.009

[5] Berlutti F, Pantanella F, Natalizi T, Frioni A, Paesano R, Polimeni A, Valenti P. Antiviral properties of lactoferrin--a natural immunity molecule. Molecules. 2011 Aμg 16; 16(8): 6992-7018. https://doi.org/10.3390/molecules16086992

[6] Xiao HY, Lan XY, Zhang PH. Research progress on the biological functions of lactoferrin. China Dairy Cattle. 2021(04): 46-50. https://doi.org/10.19305/j.cnki.11-3009/s.2021.04.011

[7] Brock JH. Lactoferrin--50 years on. Biochem Cell Biol. 2012 Jun; 90(3): 245-51. https://doi.org/10.1139/o2012-018

[8] Habault J, Poyet JL. Recent Advances in Cell Penetrating Peptide-Based Anticancer Therapies. Molecules. 2019 Mar 7; 24(5): 927. https://doi.org/10.3390/molecules24050927

[9] Morris MC, Depollier J, Mery J, Heitz F, Divita G. Nat Biotechnol. A peptide carrier for the delivery of biologically active proteins into mammalian cells. 2001 Dec; 19(12): 1173-6. https://doi.org/10.1038/nbt1201-1173

[10] Hu JW, Chen JJ, Tu W, et al. Toxicological safety study of lactoferrin bovine colostrum granules. Anhui Agricultural Science. 2012, 40(12): 7195-7198. https://doi.org/10.13989/j.cnki.0517-6611.2012.12.041

[11] Dai YN, Yang XZ, Wang JW, et al. Safety evaluation of recombinant human lactoferrin as a new raw material for cosmetics. Chinese Journal of Health Laboratory Technology, 2017, 27(13): 1851-1854+1862.

[12] Biernbaum EN, Gnezda A, Akbar S, Franklin R, Venturelli PA, McKillip JL. Lactoferrin as an antimicrobial against Salmonella enterica and Escherichia coli O157:H7 in raw milk. JDS Commun. 2021 Mar 12; 2(3): 92-97. https://doi.org/10.3168/jdsc.2020-0030

[13] Ding B, Chen Z. Molecular interactions between cell penetrating peptide Pep-1 and model cell membranes. J Phys Chem B. 2012 Mar 1; 116(8): 2545-52. https://doi.org/10.1021/jp209604m

[14] Baker EN, Baker HM. Molecular structure, binding properties and dynamics of lactoferrin. Cell Mol Life Sci. 2005 Nov; 62(22): 2531-9. https://doi.org/10.1007/s00018-005-5368-9

[15] Ekins A, Khan AG, Shouldice SR, Schryvers AB. Lactoferrin receptors in gram-negative bacteria: insights into the iron acquisition process. Biometals. 2004 Jun; 17(3): 235-43. https://doi.org/10.1023/b:biom.0000027698.43322.60

[16] Drobni, P.; Naslund, J.; Evander, M. Lactoferrin inhibits human papilloma virus binding and uptake in vitro. Antiviral Res. 2004, 64, 63-68. https://pubmed.ncbi.nlm.nih.gov/15451180/

[17] Mistry N, Drobni P, Näslund J, Sunkari VG, Jenssen H, Evander M. The anti-papillomavirus activity of human and bovine lactoferricin. Antiviral Res. 2007 Sep; 75(3): 258-65. https://doi.org/10.1016/j.antiviral.2007.03.012

[18] Zhang P, Moreno R, Lambert PF, DiMaio D. Cell-penetrating peptide inhibits retromer-mediated human papillomavirus trafficking during virus entry. Proc Natl Acad Sci U S A. 2020 Mar 17; 117(11): 6121-6128. https://doi.org/10.1073/pnas.1917748117

[19] Shahbazi S, Bolhassani A. Comparison of six cell penetrating peptides with different properties for in vitro and in vivo delivery of HPV16 E7 antigen in therapeutic vaccines. Int Immunopharmacol. 2018 Sep; 62: 170-180. https://doi.org/10.1016/j.intimp.2018.07.006

[20] Zhang P, Monteiro da Silva G, Deatherage C, Burd C, DiMaio D. Cell-Penetrating Peptide Mediates Intracellular Membrane Passage of Human Papillomavirus L2 Protein to Trigger Retrograde Trafficking. Cell. 2018 Sep 6; 174(6): 1465-1476.e13. https://doi.org/10.1016/j.cell.2018.07.031

[21] Granadillo M, Vallespi MG, Batte A, Mendoza O, Soria Y, Lµgo VM, Torrens I. A novel fusion protein-based vaccine comprising a cell penetrating and immunostimulatory peptide linked to human papillomavirus (HPV) type 16 E7 antigen generates potent immunologic and anti-tumor responses in mice. Vaccine. 2011 Jan 29; 29(5): 920-30. https://doi.org/10.1016/j.vaccine.2010.11.083