Master's Thesis

Development of IL15-FlaB Fusion Protein as a New Generation Therapeutic Cancer Vaccine Adjuvant

Department of Biomedical Sciences
Chonnam National University Medical School

Le Duy Hoang

February 2023

Development of IL15-FlaB Fusion Protein as a New Generation Therapeutic Cancer Vaccine Adjuvant

Department of Biomedical Sciences

Chonnam National University Medical School

Le Duy Hoang

Supervised by Professor Shee Eun Lee

A dissertation submitted in partial fulfillment of the requirements for the

Master of Research in Biomedical Sciences.

Committee in Charge:

Joon Haeng Rhee _____

Jae Ho Cho _____

Shee Eun Lee _____

February 2023

CONTENTS

List of Figures

List of Tables

Development of IL15 – FlaB Fusion Protein as a New Generation Therapeutic Cancer Vaccine Adjuvant

LE DUY HOANG

Department of Biomedical Sciences

Chonnam National University Medical School

(Supervised by Professor Shee Eun Lee)

(Abstract)

Cancer vaccine is one of the major modalities for cancer immunotherapy. To overcome the low immunogenicity of tumor antigens, potent adjuvants should be incorporated to elicit strong anti-tumor immune responses. In previous studies, we reported that *Vibrio vulnificus* flagellin (FlaB) has strong adjuvant activities that potentiate antigen-specific $CD8^+$ T cell immune responses through TLR-5 activation and polarizes the M2 to M1 macrophages in the tumor microenvironment. IL-15 is a promising anticancer agent to promote the differentiation and expansion of NK, NKT, $CD8^+$ T cells, and memory $CD8^+$ T cells. To take advantage of both molecules, we engineered a fusion protein composed of IL-15 and FlaB (IL15-FlaB) and tested its adjuvant activity for a therapeutic cervical cancer vaccine. The IL15-FlaB fusion protein maintained the TLR5-mediated NF-κB activity of FlaB. TC-1 tumor-bearing mice vaccinated with E7 plus IL15-FlaB fusion protein significantly enhanced antitumor effects compared to E7 plus FlaB vaccination. Taken together, an engineered IL-15-FlaB fusion protein would serve as a potent adjuvant for therapeutic cancer vaccines.

1. Introduction

Conventional modalities for cancer treatments are surgery, radiotherapy, chemotherapy, and targeted therapy. However, these treatments frequently fail to completely eliminate tumors. Immunotherapy, which makes use of immune cells to attack and eliminate cancerous cells, showed good track records in treating some cancer types. Thus, it has become the fifth pillar of cancer treatment. Although immunotherapy showed good track records in treating some cancer types, immunotherapy still has limitations, especially in tumors that have few tumor-infiltrating lymphocytes, termed "immunologically cold". Thus, many patients with immunologically cold tumors have poor treatment outcomes after immunotherapy [1]. To overcome this problem, cancer vaccines have been suggested as a modality for priming T cells and modulating tumor microenvironment in favor of anticancer effects. Additionally, cancer vaccine is safe and easy to produce, it is also easily loaded onto MHC class I molecules. However, the low immunogenicity of tumor antigens is a critical reason for the limited success of cancer vaccines in treating immunologically cold tumors [2]. To increase the immunogenicity of the antigens, oncolyticvirusess, dendritic cells, and adjuvants have been tried [3–5]. Among these three approaches, adjuvants show their own strength to enhance anti-tumor response and moderating a durable immune response, especially in immunocompromised patients [6].

In our previous studies, flagellin, a TLR-5 agonist, from *Vibrio Vulnificus* (FlaB), showed a potent adjuvant activity in vaccines for infectious diseases and cancer [7–11]. For cervical cancer, FlaB stimulates TLR-5 signaling pathways and boosts antigen-specific CD8 T cell responses in subcutaneous and orthotopic tumor models [7,11]. Furthermore, FlaB-secreted bacteria also increase anti-tumor activities and change TME by polarizing macrophage phenotypes from M2 to M1 [12]. Nonetheless, cancer vaccines adjuvanted with FlaB have not shown the durability of the T cell response which is responsible for long-lasting immunity against tumors. To address this issue, we suggest including interleukin-15 in the vaccine formula to elicit durable antitumor effects.

Interleukin-15 (IL-15) is considered as a potential anti-cancer agent because of its similar efficacy with interleukin-2 (IL-2), an approved drug by FDA for several cancer treatments. Although IL-15 shares alike structure with IL-2, IL-15 neither causes the activation-induced cell death of CD8+ effector T cells nor enriches T regulatory cells in TME which could result in diminishing anti-tumor responses. Moreover, IL-15 has the potential to promote the proliferation of NK cells, NKT cells, CD8+ T cells, and memory phenotype CD8+ T cells [13–15].

To take advantage of both molecules and exploit anti-tumor effects from innate immunity and adaptive immunity, we tested whether the combination of IL-15 and FlaB in fusion protein (IL15-FlaB) could enhance the therapeutic treatment of cancer vaccine against cervical cancer. *In vitro*, we tested the proper functions of this fusion protein by NF-κB stimulation assays for FlaB domain and CTLL-2 cell proliferation assays for IL-15 domain. To test *in vivo* effects, we used a therapeutic cervical cancer vaccine consisting of E7 antigen and IL15-FlaB fusion protein on the TC-1 tumor mouse model.

2. Materials and methods

A. Plasmid construction of IL15-FlaB fusion protein

To produce fusion protein of IL-15 and FlaB, we used pET30a$^+$ plasmid as the vector to express the protein. Between IL-15 and FlaB, we introduced furin cleavage sites, a cleavable linker to separate IL15 and FlaB domains in vivo. For accessibility by furin protease, three times repeated furin cleavage sites were used. The codon-optimized sequences of IL-15, furin cleavage sites, and FlaB were fused by overlapping PCR with the following primer sets (Table 2). In addition, appropriate primers were designed to insert suitable restriction site overhangs (Table 2). The resulting plasmid named pET30a$^+$::IF contains IL15-FlaB DNA sequence which was confirmed by Sanger sequencing via Macrogen Co., Republic of Korea.

Table 1. List of bacterial strains and plasmids in this research

Bacterial Strains	**Description**	**Source**
Escherichia coli (E.coli)		
BL21	Non-T7 expression, *fhuA2*	Laboratory collection
DH5α	F recA1; restriction negative	Laboratory collection
Plasmids		
pET30a$^+$	pBR322 Ori, Kanamycin resistance, f1 Ori, T7 promoter, Lac1	Novagen, 69909 – Merck Millipore

Table 2. PCR Primers used in this study

Symbol	Direction	Sequences (5' to 3')
mIL15 NdeI F	Forward	GGGAATTCCATATGAATTGGATCGATGTACGC
3xfurin IL-15 FlaB F	Forward	ATGTTCATTAACACGTCACGCGTGAAACGCCG CGTGAAACGCCGCGTGAAACGCATGGCAGTGA ATGTAAAT
3xfurin IL-15 FlaB R	Reverse	ATGTTCATTAACACGTCACGCGTGAAACGCCG CGTGAAACGCCGCGTGAAACGCATGGCAGTGA ATGTAAAT
FlaB HindIII R	Reverse	CAGCGCTAAGTCTACTAGGCAAGCTTGGG

The underlined nucleotides are restriction recognition sites.

Specific restriction enzymes are mentioned in the symbol of the primer, if necessary.

B. Expression and purification of IL15- FlaB fusion protein

Competent *E.coli* BL21 was transformed with pET30a^{+}::IF by heat shock method. Then, we added 1mM isopropyl-β-D-thiogalactoside (IPTG) in mid-log phase cultures of BL21 harboring pET30a^{+}::IF to induce protein expression. The culture media were kept in shaking machine overnight at 18^{o}C. After expression, bacteria were pelleted by centrifugation, at 8,000 rpm for 10 minutes. To prepare the lysate for purification, bacterial pellets were dissolved in lysis buffer (pH 8, 10 mM imidazole, 20 μM phenylmethylsulfonyl fluoride, 50 mM NaH_2PO4, 300 mM NaCl, 0.1% Triton X-100, 0.1% Tween). Then, the bacterial lysate was sonicated for 15 minutes on an ice bed, and pulsed after 5 seconds. The cell-free supernatant was collected after centrifugation for 30 minutes at 18,000 rpm. Next, the recombinant protein of His-tagged IL15-FlaB was purified by affinity column chromatography using Ni-NTA agarose beads (Qiagen,

Hilden, Germany). According to the manufacturer's protocol, we increased imidazole concentration from 10 mM to 50 mM for washing buffer and then eluted protein at 250 mM imidazole. In the washing buffer, we also added 0.1% Triton X-114 to remove lipopolysaccharide (LPS). Next, we confirmed the purity of the recombinant protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The level of endotoxin of the protein was examined by gel-clotting Endosafe LAL kit (Charles River Endosafe, Charleston, SC). The LPS level was kept below FDA guidelines (less than 0.15 EU/30g per mouse). The concentration of purified protein was determined by Bradford dye-binding assay (Bio-Rad Laboratories).

C. Western blot analysis

To confirm the fusion protein, we used western blot analysis to detect the recombinant protein. After running SDS-PAGE, the gel was transferred onto nitrocellulose membrane (Amersham, 10600004). The specific murine antibodies for FlaB or IL-15 were then incubated with the membrane for 2 hours at room temperature to detect corresponding proteins. After washing by PBS with Tween 0.05%, the membrane was incubated for 1 hour with horseradish peroxidase (HRP)-conjugated polyclonal clonal rabbit anti-mouse immunoglobulins (Daiko, P0260). Finally, we used chemiluminescent HRP Substrate (Millipore, WBKLS0500) to visualize proteins.

D. Digestion of IL15-FlaB fusion protein with furin protease

In our construct, we introduced the cleavable linker between IL-15 and FlaB. To test the efficacy of cleavage sites, we incubated the IL15-FlaB recombinant protein with commercial furin protease for 36 hours at 37^{o}C. The reaction solution after cleavage was loaded on SDS-PAGE gels to separate the fragments. We confirmed the cleaved fragments by SDS-PAGE and western blot analysis (described above).

E. Evaluation of TLR5-dependent NF-κB activity

To confirm the biofunctional activity of IL15-FlaB fusion protein, we measured TLR-5-stimulating activity by using NF-κB reporter assay. First, we seeded 2x10^{5} HEK293T cells/ well on a 24-well plate. After 1 day, we transfected these cells with p3x Flag-hTLR5 (100 ng/well), pNFκB-Luc (100 ng/well), and 50 ng/well of pCMV-β-Gal using 5 µl/well of Effectene (Qiagen, 301427). After 16 hours, the cells were treated with endotoxin-free proteins (200 ng FlaB, 265 ng IF) for 18 hours. The luciferase signal was measured by luminometer (Berthold, Lumat-Plus LB 96V) and normalized with the β-galactosidase signal. PBS-treated group was set up as the control group.

F. Determination of CTLL2 proliferation assay

To determine whether the fusion protein maintains the functions of IL-15, we measured the ability of recombinant protein in stimulating CTLL2 cell proliferation. In this assay, we seeded $3x10^4$ CTLL2 cells in each well of 96-well U-shape plate. The plate was incubated in incubator at 37°C, 5% CO_2 overnight. Then, we diluted IL-15 and fusion protein in serum-free RPMI 1640 media and in two-fold serial dilution. Next, we continued to incubate the plate for 72 hours to stimulate cell proliferation. On the final day, we used multiple pipette and reservoir to add 10µl of CCK-8 (CK04, Dojindo) into each well. After that, the plate was incubated for 3 hours at 37°C. The optical signal was read by microplate reader (Molecular Devices Corp., Menlo Park, CA) at 450 nm.

G. Tumor implantation, vaccination, tumor measurement, and single cell suspension

TC-1 cells originate from mouse lung epithelial cells and are stably transfected with early proteins E6 and E7 of human papilloma virus 16 (HPV-16). Tumors were implanted in seven-week-old C57BL/6 mice (ORIENT, Korea) by injecting subcutaneously with $5x10^4$ TC-1 cells. Once tumors reached 3-5 mm^3, mice were separated randomly into different groups corresponding to different treatments. Different formulas of therapeutic vaccines were then injected peritumorally according to the schedule in Figure 5A. Tumor volume was measured twice a week by a caliper and calculated with formula:

$$V = L \times W \times H/2 \ (mm^3)$$

(V: Tumor volume – mm^3, L: length of the tumor, longer diameter– mm, W: width of the tumor, shorter diameter – mm, H: height of the tumor – mm)

For the immunological mechanism study, TC-1 tumor-bearing mice were treated with the same schedule. We sacrificed the mice and took spleen and tumor draining lymph nodes (TDLN) for single cells at one week after the last vaccination. The single cells were diluted in complete

RPMI 1640 media and filtered through 40μm strainers. To remove red blood cells, the cell suspension with $1x10^6$ cells was mixed with 1 ml ACK Lysing Buffer (Gibco, A10492) and then filtered again.

H. Tetramer staining

To determine tumor-specific $CD8^+$ T cells, we collected peripheral blood from TC1-tumor-bearing mice at one week after the third immunization and stain with HPV16 H-2Db-RAHYNIVTF tetramer reagent (TB-5008-1, MBL) following the manufacturer's instructions. The combination of tetramer staining and marker CD8α was used to detect E7-specific $CD8^+$ T cells.

I. Flow cytometry (FACS)

After single-cell suspensions, $1x10^6$ cells per sample (spleen, TDLN) were prepared for flow cytometry analyses. These cells were stained with Ghost Dye (Cell Signaling, 59863S) for live/dead detection and surface markers with respective fluorescence: anti- NK1.1-FITC, anti-CD45-PE, anti-TCRβ-PB to detect NK, NKT cells; anti-CD8-FITC, anti-CD4-PE, anti-CD44- PE-Cy7, anti-CD3-PB to detect T cell subpopulations. After staining for 30 minutes at 4^oC, the cells were washed and fixed with 4% formaldehyde until analysis. Data acquisition was performed on BD FACS Canto flow cytometer (BD Biosciences) and analyzed with Flowjo software (Tree Star, Ashland, OR).

3. Result

A. Plasmid construction and protein purification

pET30a^{+}::IF plasmid containing fused IL15-FlaB with furin cleavage sites was verified by Sanger sequencing. Final plasmid, gene organization, and putative structural simulation were shown in Figure 1. The recombinant protein was loaded onto nickel gravity-flow chromatography and purified from the cell lysate. The purified protein was determined by SDS-PAGE. It showed that the targeted protein compensated over 90% of final products with the expected size in the gel (FlaB – 42 kDa, IL-15 – 12.7 kDa, IF – 55.8 kDa,) (Figure 2A). The fusion protein IF was recognized by specific antibodies of FlaB and IL-15 (Figure 2B & C). These results indicated that the fusion protein of Il-15 and FlaB was successfully expressed and purified.

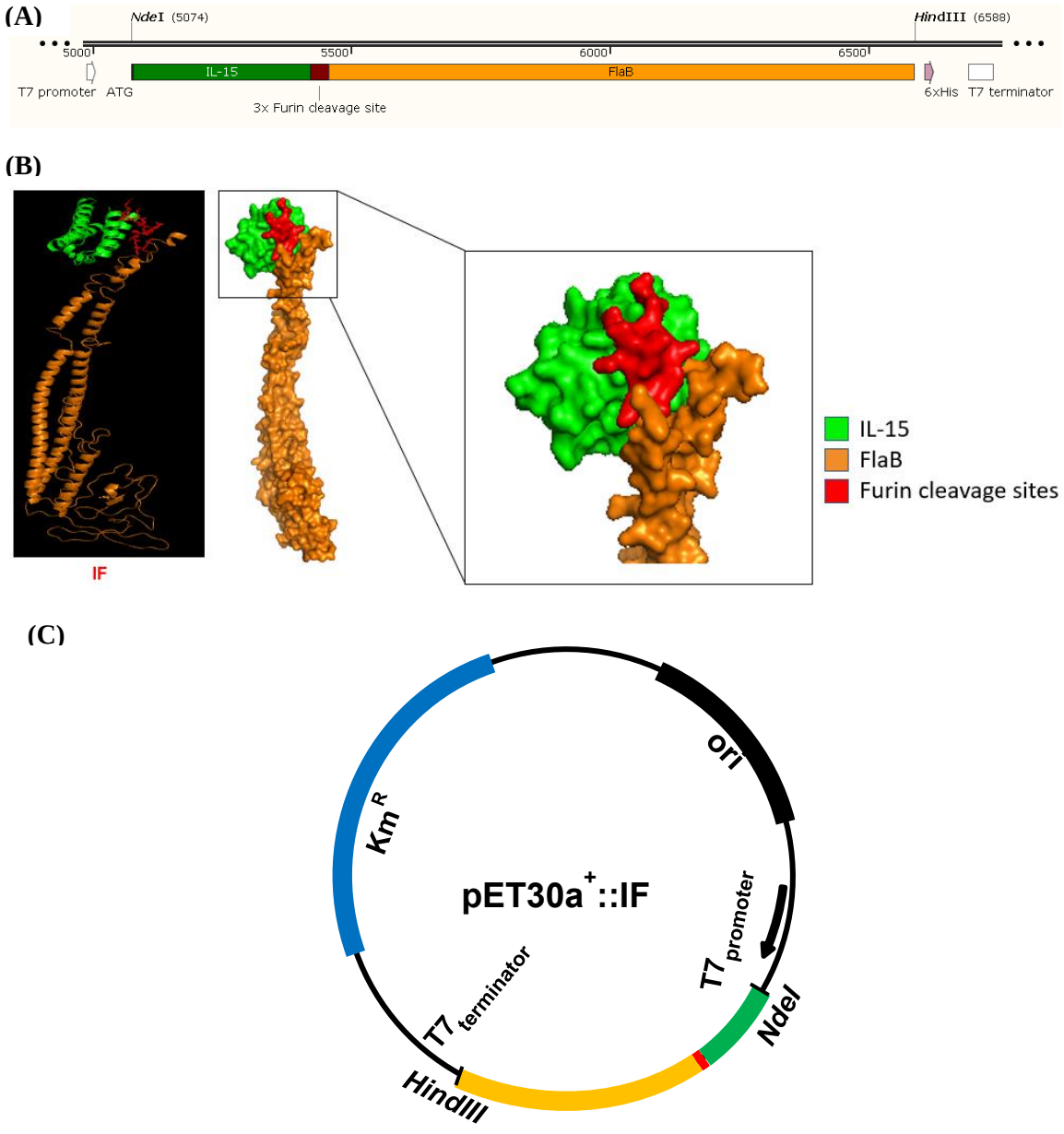


Figure 1. Plasmid constructs and structural simulation of IL15-FlaB fusion protein

A. Fusion strategy

B. Putative structural simulation of fusion protein IF

C. Plasmid construct of IL-15 and FlaB recombinant protein (pET30a^{+}::IF)

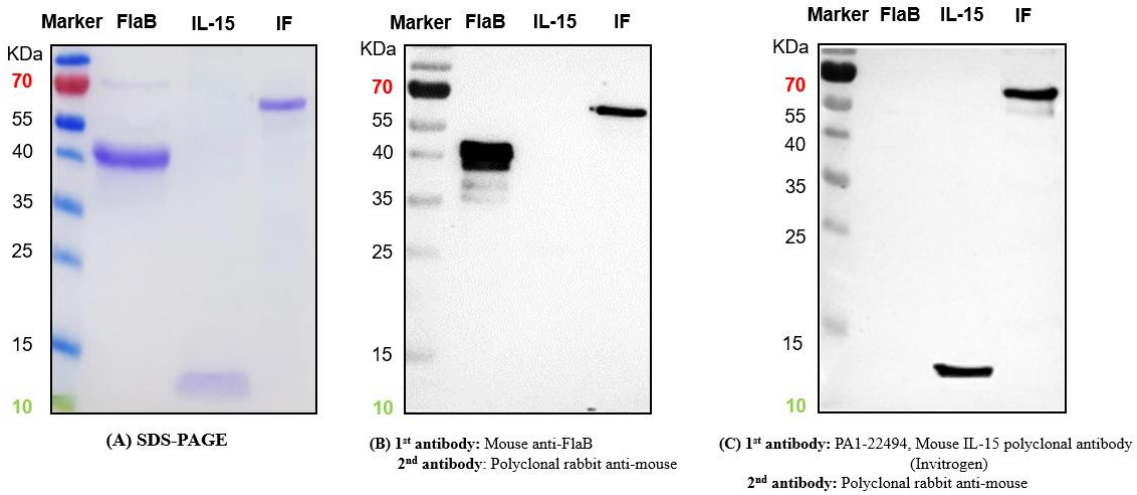


Figure 2. SDS-PAGE and western blot analysis of recombinant protein after purification

A. Recombinant proteins on SDS-PAGE gel after purification
B. Western blot analysis with mouse anti-FlaB antibodies
C. Western blot analysis with mouse anti-IL-15 antibodies

B. *In vitro* digestion of cleavable linker

Due to different target cells of IL-15 and FlaB, we introduced cleavable linker between these two partners to separate them *in vivo*. We inserted three-time repeated furin cleavage sites between IL-15 and FlaB. To test the cleavable ability of this linker, we incubated the recombinant protein with commercial furin protease following the manufacturer's instructions. After incubation at 37°C for 36 hours, new protein fragments appeared under our original recombinant protein band (Figure 3A). We confirmed those bands by western blot with anti-FlaB and anti-IL-15 antibodies and they are well detected (Figure 3B & C). It demonstrates that the fusion protein IF was cleaved by furin protease as a hint for the separation of two parts, IL-15 and FlaB, *in vivo*.

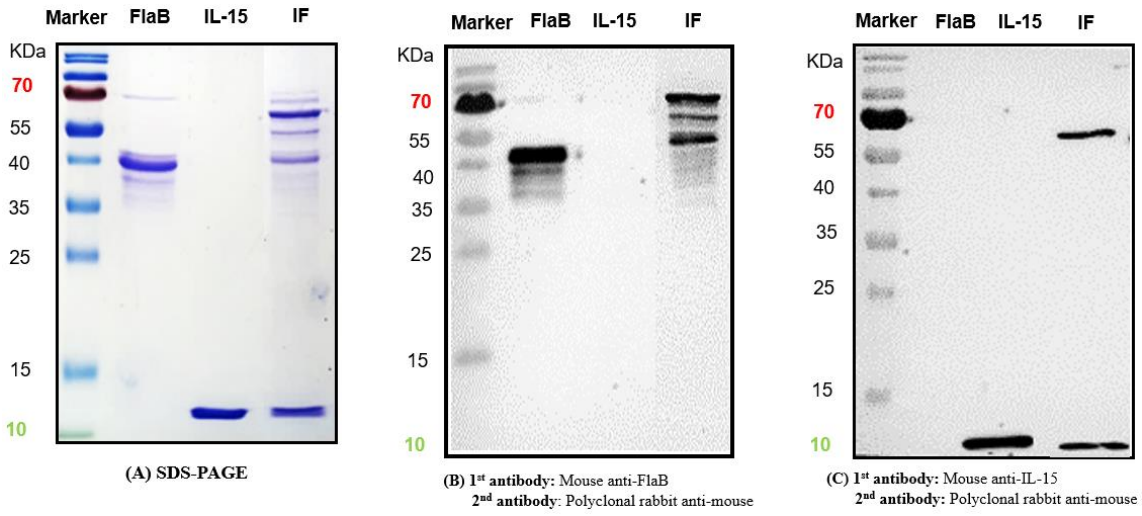


Figure 3. Test cleavable linkers by furin protease *in vitro*

A. SDS-PAGE analysis of recombinant protein after incubation with furin protease for 36 hours at 37°C;

B. Western blot analysis with anti-FlaB antibodies to identify cleaved fragments

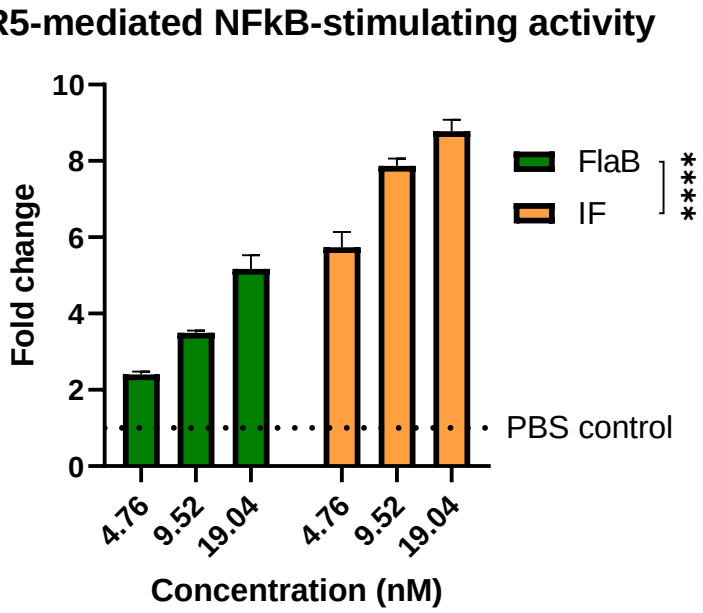
C. Western blot analysis with anti-IL-15 antibodies to identify cleaved fragments

C. Biofunctional activities of IL15-FlaB fusion protein

To test whether the recombinant protein IF maintains the TLR-5-dependent activation, we added the protein IF into NF-κB signaling reporter assay. The fusion protein exhibited dose-dependent NF-κB activation. Moreover, compared to FlaB alone, this fusion protein showed higher activities (Figure 4A).

For activating function of IL-15, we added the recombinant protein in serial two-fold dilution with CTLL-2 cells, which present IL-2Rα, IL-2/IL-15Rβ and γ common chain, and measured cell proliferation. Although commercial IL-15 stimulates cell proliferation, the recombinant protein IF did not show the comparable activation activity (Figure 4B).

(A)



(B)

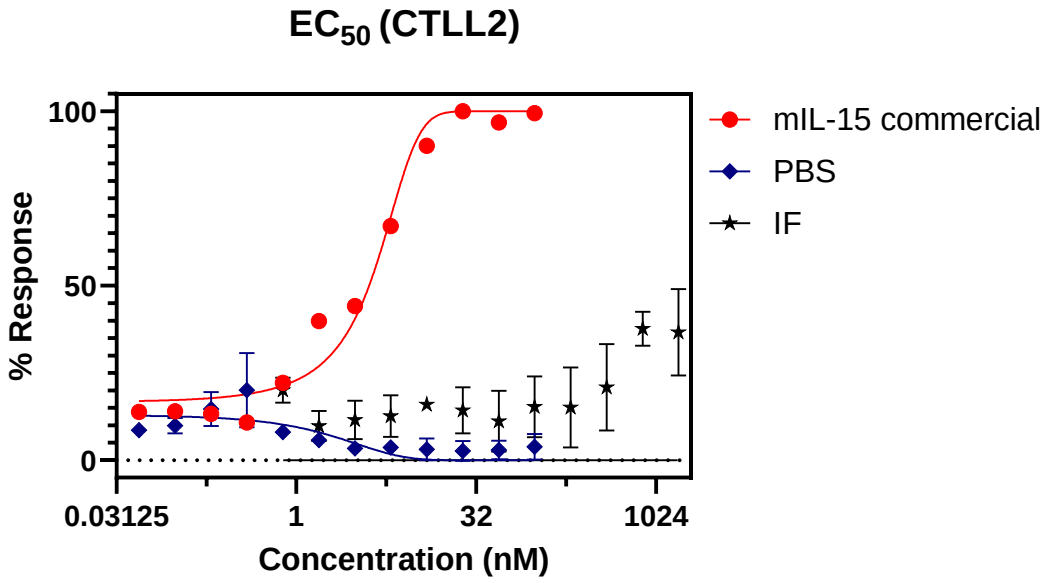


Figure 4. Functional assays for FlaB and IL-15 bioactivities

A. TLR-5 mediated NF-κB signaling assay for FlaB functions

B. CTLL2 proliferation assay for IL-15 functions

D. IL15-FlaB fusion protein potentiated therapeutic cancer vaccine by inducing tumor suppression and prolonging survival in tumor mouse model

We next evaluated the adjuvant activity of recombinant protein on therapeutic outcome using syngeneic mouse tumor model. Eight days after TC-1 implantation, we began injecting tumor-bearing mice with the E7 antigen (E7ΔNLS) plus fusion protein of IL-15 and FlaB (IF). In our previous study, we proved that E7 with nucleus localizing sequence deletion (E7ΔNLS) was an optimal antigen for this tumor mouse model in combination with FlaB [10]. In this experiment, cancer vaccine using E7ΔNLS antigen alone also showed tumor suppression effect compared to non-vaccinated group. When E7ΔNLS was co-administered with FlaB, the antitumor effect was enhanced. Furthermore, at early timepoint (until day 30 post implantation), IL-15 and FlaB fusion protein (IF) and E7ΔNLS co-injection showed significant inhibition of tumor growth in comparison with E7ΔNLS plus FlaB group (Figure 5C). In later timepoint, although therapeutic vaccines increased survival of treated animals in IF plus E7ΔNLS group, the difference between this group and E7ΔNLS plus FlaB group was not noticeable (Figure 5D). These results indicate that fusion protein of IL-15 and FlaB potentiate therapeutic effects of cancer vaccine and show as an effective adjuvant.

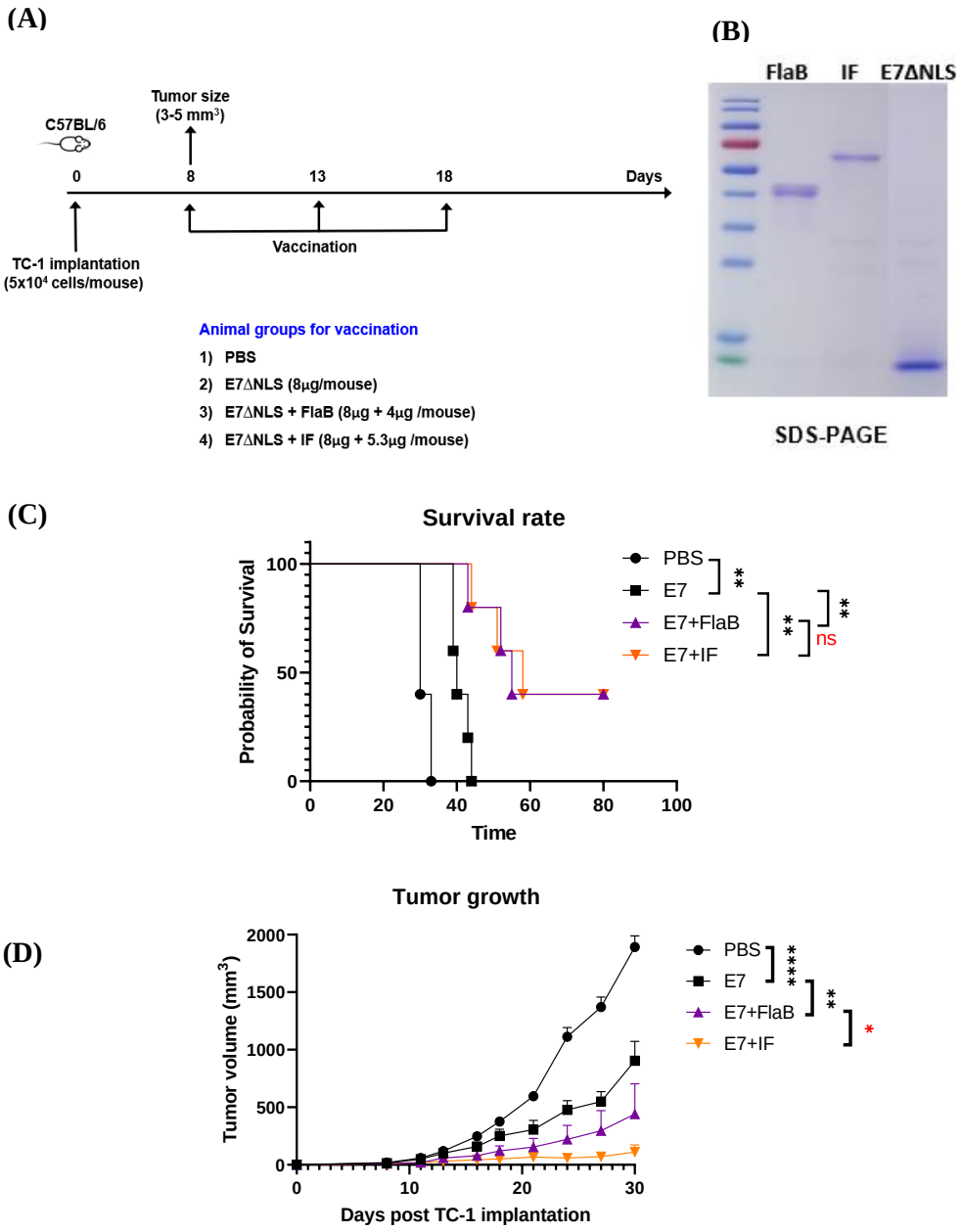


Figure 5. Vaccination schedule, tumor growth and survival rate of treated mice

A. Immunization schedule with TC-1 mouse model

B. SDS-PAGE of proteins used in vaccination

C. Tumor growth at day 30 post implantation

D. Survival rate until day 80 post implantation

E. Tumor-bearing mice treated with IL15-FlaB increased $CD8^+$ T cells and potentiated antigen-specific effects in TDLNs

To clarify the reason behind the antitumor effect at early timepoint, we identified $CD8^+$ T cell populations in TDLNs to see the changes between FlaB and fusion protein IF groups in co-administration with E7ΔNLS. As a result, co-administration of E7ΔNLS and IF significantly increased higher amount of $CD8^+$ T cells in tumor draining lymph nodes compared to FlaB adjuvanted group (Figure 6B). It demonstrates that the fused IL15-FlaB platform presents better advantages in recruiting effector immune cells compared to FlaB only group. In systemic responses, three times of vaccination did not show significant difference between FlaB and IF group (Figure 6C).

Furthermore, we checked the tumor-specific T cells generated by therapeutic cancer vaccines by tetramer staining. One week after the last immunization, whole blood was collected to identify E7-specific $CD8^+$ cells (Figure 6A). As a result, E7ΔNLS treated group showed enhancement of E7-specific CD8 in blood compared to non-treated mice (Figure 6D&E). Furthermore, FlaB adjuvanted group exhibited higher amount of E7-specific $CD8^+$ T cells. Noticeably, this figure for IF plus E7ΔNLS group was significantly increased compared to FlaB plus E7ΔNLS group (Figure 6D&E). These results demonstrate the improvement of immune responses when IL-15 and FlaB fusion protein are used as an adjuvant in cancer vaccine.

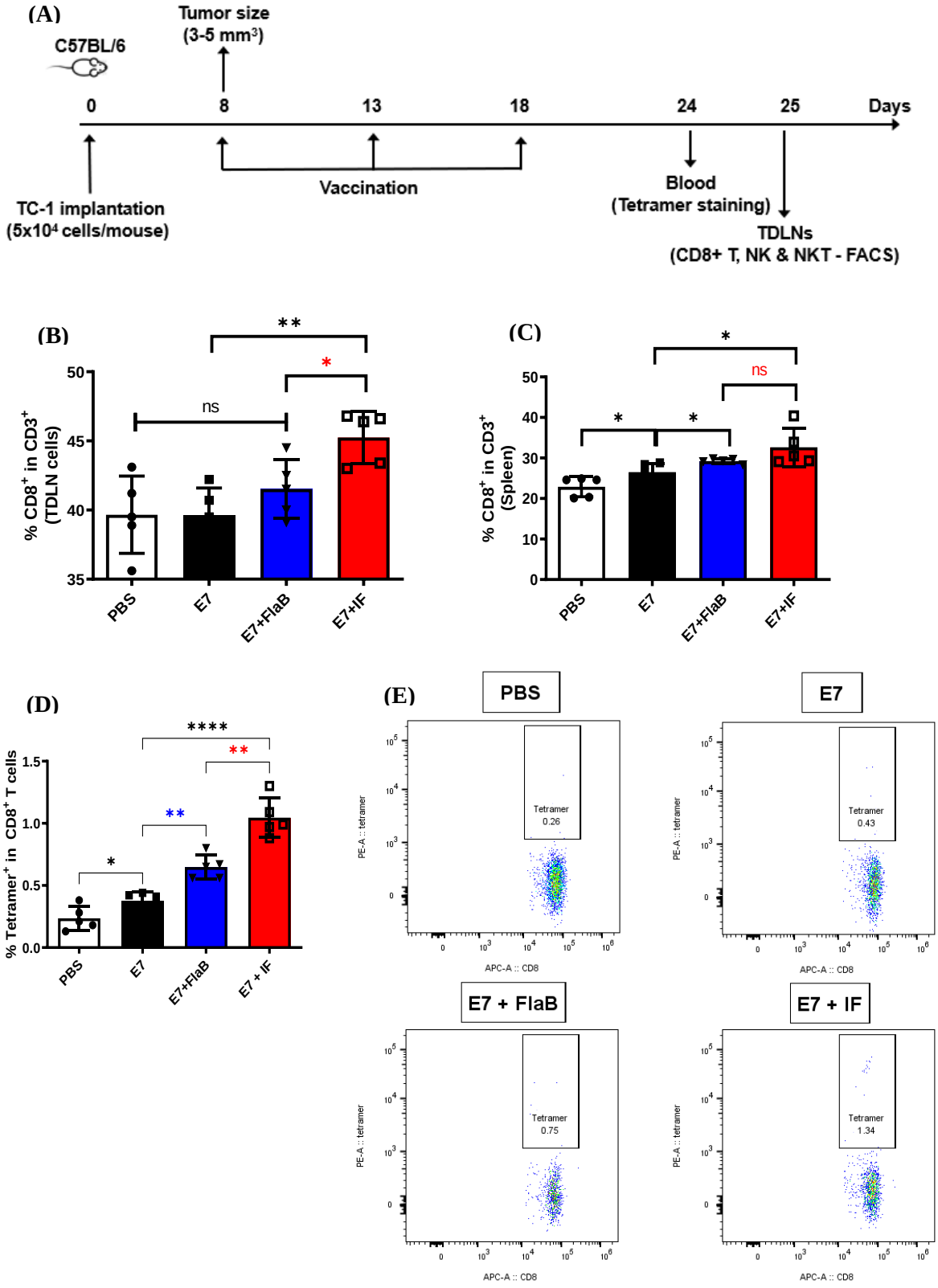


Figure 6. Determination of CD8⁺ T cells in tumor draining lymph nodes and spleen

Figure 6. Determination of $CD8^+$ T cells in tumor draining lymph nodes and spleen

A. Immunization schedule for immunological study

B. $CD8^+$ T cells in TDLNs

C. $CD8^+$ T cells in spleen

D. $Tetramer^+$ percentage in $CD8^+$ T cell in TDLNs

E. Representative dot plot graphs showing E7-specific $CD8^+$ T cells

F. IL15-FlaB fusion protein increased NK and NKT cell recruitment in TDLNs

Since IL-15 is a pleiotropic cytokine with roles in innate and adaptive immunity, we investigated further on other IL-15 targeting cells, such as NK and NKT cells, upon the therapeutic treatments. In the similar pattern with $CD8^+$ T cells, the percentage of NK and NKT cells in TDLNs increased significantly in IF plus E7ΔNLS group compared to FlaB plus E7ΔNLS group (Figure 7A). In addition, there was no significant difference of NK and NKT cells in spleen between IF and FlaB treatment groups (Figure 7B). These results provide more evidence about the better effects of fusion protein of IL-15 and FlaB over FlaB alone in recruiting immune cells in TDLNs.

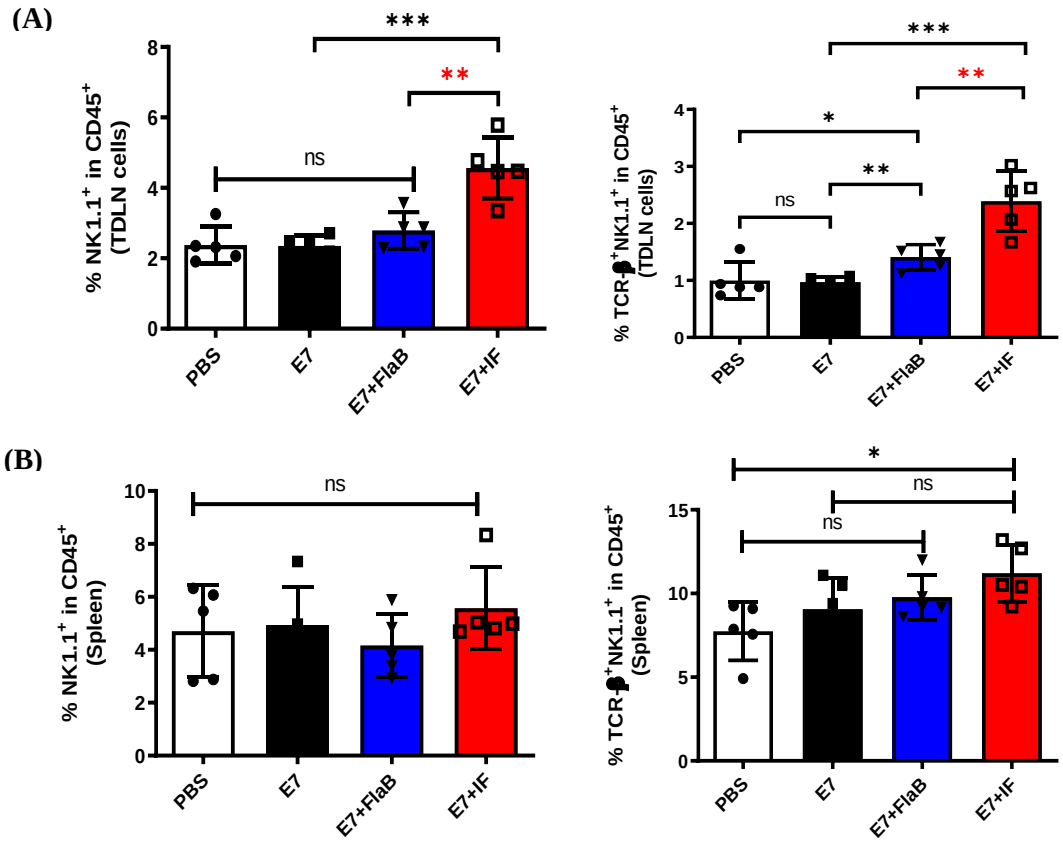


Figure 7. Determination of NK and NKT cells in TDLNs and spleens by FACS analysis

A. Percentage of NK and NKT cells in TDLNs

B. Percentage of NK and NKT cells in spleen

4. Discussion

Adjuvant plays an essential role in the development of cancer vaccine to boost the tumor-specific antitumor effects. In this research, we report that the fusion protein of IL-15 and FlaB served as a potential adjuvant for therapeutic cancer vaccine to elicit strong immune responses against tumors. To take advantages of both IL-15 and FlaB, an all in one combination or fusion platform would be more convenient solution economically. Since IL-15 and FlaB target different immune cells, we engineered these two arsenals of IL15 and FlaB with cleavable linker to separate these two domains after immunization. Here, we introduced furin cleavage sites as cleavable linker between IL15 and FlaB. Furin is a membrane associated enzyme which is paired basic amino acid to cleave the two domains to separate after inoculated into *in vivo*. The expectation is that the fusion of IL-15 and FlaB will encounter with immune cells then FlaB domain will bind to TLR5 on cells' surface and be internalized and IL15 domain will be released outside the cells due to cleavage of furin at the cell membrane. Thus, FlaB will activate immune cells via TLR5 or NLRC4-dependent pathways while IL15 will separately activate other NK cell, or CD8 T cells, enhancing antitumor effects. With that concept, we successfully fused N-terminus of FlaB with IL-15 (IF) and then produced it in *E.coli* expressing system (Figure 2). In addition, this fusion protein can be cleaved by furin protease (Figure 3).

In NF-κB reporter assay, the fusion protein significantly enhanced the TLR5-mediated signalling pathway compared to FlaB alone (Figure 4A). However, the IL-15 function of fusion protein IF was decreased compared to commercial IL-15 (Figure 4B). This *in vit*ro reduction maybe due to the proper folding of FlaB which causes the steric hindrance and affects IL-15 presentation to CTLL-2 cells. CTLL-2 is recognized as gold standard in IL-2 and IL-15 functional assay because these cells exhibit IL-2Rα, IL-2/IL-15Rβ and common γ chain [16]. However, previous studies proved that the main mechanism of IL-15 functions is based on the trans-

presentation of IL-15 from producing cells to targeting cells which display IL-2/IL-15Rβ and common γ chain [17,18]. To analyse the IL-15 functions of the recombinant protein, we should further try with other suitable cell lines and test them *in vivo*. These results show that after purification, the recombinant protein of IL-15 and FlaB folded properly (Figure 1B) and show biofunctional activity via TLR-5 signaling (Figure 4A).

Next, we proceed to evaluate the therapeutic effects of the fusion protein IF in tumor mouse model. At the early timepoint, the tumor-bearing mice vaccinated with IF plus E7ΔNLS enhanced immunological responses against E7, significantly inhibited tumor growth and compared to FlaB plus E7ΔNLS treated group. However, IL-15 and FlaB fusion protein group showed the comparable effects with FlaB plus E7ΔNLS in long-term antitumor effects as the survival rates were similar. The reason for early antitumor effects could be the limited half-life of IL-15 in the body. In some clinical trials, IL-15 is administered on daily basis or weekly basis for long time [19–22]. For future study, we will test new schedule for cancer vaccine using IF to extend the long-term immune responses against tumors.

In addition, to evaluate the mechanism behind antitumor effects, we profiled the targeting cells of IL-15 and FlaB to see the immunological changes between treatment groups. Vaccine regiment contains fusion of IL-15 and FlaB (IF) potentiates E7-specific immune responses and enhances activation of CD8, NK, and NKT cells in TC-1 mouse model. From these results, we clearly see the advantages of combinational therapy of IL-15 and FlaB in recruiting the effector immune cell population in tumor draining lymph nodes, but not in systemic immunity. Taken together, tumor antigen along with IL15-FlaB fusion protein may provide a potential therapeutic platform as an effective cancer vaccine adjuvant.

However, for better stability and function of IL15-FlaB fusion protein, we need further optimization for this fusion construct. We may include an IL-15 receptor α to enhance the activity of IL-15 part and we will further test a variety of cleavable substrates for different overexpressed proteases in TME such as MMPs, serine proteases to separate FlaB and IL-15 *in vivo*.

5. References

1. Zhang Y, Zhang Z. The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications. Cell Mol Immunol. 2020;17: 807–821. doi:10.1038/s41423-020-0488-6

2. Saxena M, van der Burg SH, Melief CJM, Bhardwaj N. Therapeutic cancer vaccines. Nat Rev Cancer. 2021;21: 360–378. doi:10.1038/s41568-021-00346-0

3. Le Gall CM, Weiden J, Eggermont LJ, Figdor CG. Dendritic cells in cancer immunotherapy. Nat Mater. 2018;17: 474–475. doi:10.1038/s41563-018-0093-6

4. Park AK, Fong Y, Kim S-I, Yang J, Murad JP, Lu J, et al. Effective combination immunotherapy using oncolytic viruses to deliver CAR targets to solid tumors. Sci Transl Med. 2020;12: eaaz1863. doi:10.1126/scitranslmed.aaz1863

5. Facciolà A, Visalli G, Laganà A, Di Pietro A. An Overview of Vaccine Adjuvants: Current Evidence and Future Perspectives. Vaccines. 2022;10: 819. doi:10.3390/vaccines10050819

6. Hu Z, Ott PA, Wu CJ. Towards personalized, tumour-specific, therapeutic vaccines for cancer. Nat Rev Immunol. 2018;18: 168–182. doi:10.1038/nri.2017.131

7. Lee SE, Hong SH, Verma V, Lee YS, Duong T-MN, Jeong K, et al. Flagellin is a strong vaginal adjuvant of a therapeutic vaccine for genital cancer. OncoImmunology. 2016;5: e1081328. doi:10.1080/2162402X.2015.1081328

8. Hong SH, Byun Y-H, Nguyen CT, Kim SY, Seong BL, Park S, et al. Intranasal administration of a flagellin-adjuvanted inactivated influenza vaccine enhances mucosal immune responses to protect mice against lethal infection. Vaccine. 2012;30: 466–474. doi:10.1016/j.vaccine.2011.10.058

9. Nguyen CT, Kim SY, Kim MS, Lee SE, Rhee JH. Intranasal immunization with recombinant PspA fused with a flagellin enhances cross-protective immunity against Streptococcus pneumoniae infection in mice. Vaccine. 2011;29: 5731–5739. doi:10.1016/j.vaccine.2011.05.095

10. Puth S, Verma V, Hong SH, Tan W, Lee SE, Rhee JH. An all-in-one adjuvanted therapeutic cancer vaccine targeting dendritic cell cytosol induces long-lived tumor suppression through NLRC4 inflammasome activation. Biomaterials. 2022;286: 121542. doi:10.1016/j.biomaterials.2022.121542

11. Nguyen CT, Hong SH, Sin J-I, Vu HVD, Jeong K, Cho KO, et al. Flagellin enhances tumor-specific CD8+ T cell immune responses through TLR5 stimulation in a therapeutic cancer vaccine model. Vaccine. 2013;31: 3879–3887. doi:10.1016/j.vaccine.2013.06.054

12. Zheng JH, Nguyen VH, Jiang S-N, Park S-H, Tan W, Hong SH, et al. Two-step enhanced cancer immunotherapy with engineered Salmonella typhimurium secreting heterologous flagellin. Sci Transl Med. 2017;9: eaak9537. doi:10.1126/scitranslmed.aak9537

13. Robinson TO, Schluns KS. The potential and promise of IL-15 in immuno-oncogenic therapies. Immunol Lett. 2017;190: 159–168. doi:10.1016/j.imlet.2017.08.010

14. Berger A, Colpitts SJ, Seabrook MSS, Furlonger CL, Bendix MB, Moreau JM, et al. Interleukin-15 in cancer immunotherapy: IL-15 receptor complex versus soluble IL-15 in a cancer cell-delivered murine leukemia model. J Immunother Cancer. 2019;7: 355. doi:10.1186/s40425-019-0777-8

15. Steel JC, Waldmann TA, Morris JC. Interleukin-15 biology and its therapeutic implications in cancer. Trends Pharmacol Sci. 2012;33: 35–41. doi:10.1016/j.tips.2011.09.004

16. Wei X, Orchardson M, Gracie JA, Leung BP, Gao B, Guan H, et al. The Sushi Domain of Soluble IL-15 Receptor α Is Essential for Binding IL-15 and Inhibiting Inflammatory and Allogenic Responses In Vitro and In Vivo. J Immunol. 2001;167: 277–282. doi:10.4049/jimmunol.167.1.277

17. Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A. Dendritic Cells Prime Natural Killer Cells by trans-Presenting Interleukin 15. Immunity. 2007;26: 503–517. doi:10.1016/j.immuni.2007.03.006

18. Chirifu M, Hayashi C, Nakamura T, Toma S, Shuto T, Kai H, et al. Crystal structure of the IL-15–IL-15Rα complex, a cytokine-receptor unit presented in trans. Nat Immunol. 2007;8: 1001–1007. doi:10.1038/ni1492

19. Romee R, Cooley S, Berrien-Elliott MM, Westervelt P, Verneris MR, Wagner JE, et al. First-in-human phase 1 clinical study of the IL-15 superagonist complex ALT-803 to treat relapse after transplantation. Blood. 2018;131: 2515–2527. doi:10.1182/blood-2017-12-823757

20. Miller JS, Morishima C, McNeel DG, Patel MR, Kohrt HEK, Thompson JA, et al. A First-in-Human Phase I Study of Subcutaneous Outpatient Recombinant Human IL15 (rhIL15) in Adults with Advanced Solid Tumors. Clin Cancer Res. 2018;24: 1525–1535. doi:10.1158/1078-0432.CCR-17-2451

21. Bessard A, Solé V, Bouchaud G, Quéméner A, Jacques Y. High antitumor activity of RLI, an interleukin-15 (IL-15)–IL-15 receptor α fusion protein, in metastatic melanoma and colorectal cancer. Mol Cancer Ther. 2009;8: 2736–2745. doi:10.1158/1535-7163.MCT-09-0275

22. Desbois M, Béal C, Charrier M, Besse B, Meurice G, Cagnard N, et al. IL-15 superagonist RLI has potent immunostimulatory properties on NK cells: implications for antimetastatic treatment. Open Access. : 13.

IL15-FlaB 융합단백질을 이용한 차세대 항암백신 면역증강제 개발

LE DUY HOANG

전남대학교 대학원 의과학과

(지도교수 : 이시은)

(초록)

항암 백신이 다양한 암 치료를 위한 전도유망한 면역치료요법으로써 연구되어온 이래 종양항원의 낮은 면역원성을 극복하기 위해 강력한 면역증강제의 필요성이 점점 대두되고 있다. 선행연구를 통해서 본 연구진은 플라젤린 (FlaB)이 TLR-5 활성화를 통해 항원 특이적 CD8+ T 세포 면역반응 증가 및 종양 미세환경 내 M2 에서 M1 대식세포로의 형질전환을 유도하는 강력한 면역증강제로서의 역할을 수행한다는 사실을 밝혀냈다. IL-15 는 NK, NKT, CD8+ T 세포, 그리고 기억 면역 CD8+ T 세포의 분화 및 활성화 촉진을 통해서 항암 효과를 나타내는 것으로 보고된 바 있다. 이 두 물질의 장점을 고루 취하기 위해 FlaB 와 IL-15 로 구성된 융합 단백질을 제작하여 자궁경부암 백신 요법에서 면역증강제로서의 가능성을 검증하고자 하였다. 해당 융합 단백질은 시험관 내에서 FlaB 에 의한 TLR5-매개 NF-κB 활성화를 증가시켰다. 또한 TC-1 종양이식 생쥐 모델에서 융합 단백질에 의한 E7 항원 특이적 면역반응 증가, 종양성장 억제효과 및 생존률 증가를 관찰하였다. 이를 통해 IL15-FlaB 융합 단백질과 종양 항원의 동시 처리가 효과적인 자궁경부암 항암요법을 위한 플랫폼 구축에 유용한 단초를 제공할 것으로 사료된다.

Acknowledgement

This thesis would not have been possible without the generous assistance of my family, my supervisors, Prof. Shee Eun Lee and Prof. Joon Haeng Rhee, teachers and lab members at the Combinatorial Tumor Immunotherapy Research Center, and dear friends at Chonnam National University Medical School.

I am writing to express my sincerest gratitude and respect for Professor Shee Eun Lee's training and supervision. Under her guidance and coaching, I have steadily sharpened my skills, developed my critical thinking, and grown as a scientist. I also want to thank Professor Joon Haeng Rhee for his guidance. It is a privilege to be his student and to learn from him every day, as he has an expansive vision and a clear objective to aid patients.

Our former lab members established this study, which was specially developed and supported by Sao Puth, PhD. I would like to convey my most profound appreciation to Dr.Sao for his patience in constantly lending me a hand and his dedication to science, which has inspired me daily. In addition, Nguyen Tien Duc, PhD, a big brother, and a conscientious scientist who is up for significant challenges and finds joy in science, also made substantial contributions to this effort.

Lastly, I'd like to thank my beautiful family for always rooting for me. Without them, I would not have reached this point in my scientific career.

February 2023, South Korea

Le Duy Hoang