

DEVELOPMENT OF A NEW VACCINE TECHNOLOGY USING THE  
CARRIER/ADJUVANT PROPERTIES OF ASC SPECKS

by

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## ABSTRACT

### DEVELOPMENT OF A NEW VACCINE TECHNOLOGY USING THE CARRIER/ADJUVANT PROPERTIES OF ASC SPECKS

Pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) are sensed by the nucleotide-binding oligomerization domain-like receptor (NLR) family of proteins in the cytosol for the regulation of innate immunity responses. Certain NLRs such as NLRP3 induce the formation of inflammasome complexes. Caspase-1 is activated within the inflammasome multiprotein complex via interaction with apoptosis-associated speck-like protein (ASC). The adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) is 22 kDa cytosolic protein that contains a C-terminal caspase recruitment domain (CARD) and an N-terminal pyrin (PYD) domain. Upon stimulation, ASC combines into filaments and with many ASC molecules to form a globular structure called ASC speck. These specks can exist inside the cell or can be released to the extracellular space in order to amplify the signals such as many cytokines. In previous data of our group, artificial loading of ASC specks was shown. We showed that ASC specks in PBS remained stable at 37°C for 30 days. We have also shown the long-lasting stability of ASC specks inside the spleen of mice. The aim of the thesis is to purify ASC specks with antigen epitopes and achieve vaccination using ASC specks. After stimulation of THP-1 macrophages with purified ASC specks, we showed an increase in the secretion of certain inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ . Besides, tH5-ASC immunized C57BL/6 mice had higher anti-H5 IgG levels compared to other groups of immunized mice. Lastly, we showed that i.p. injected tOVA-ASC specks may go to the EG7-OVA thymoma tumor in C57BL/6 mice and cause complete eradication or reduction in tumor size.

## ÖZET

### ASC ZERRECİKLERİNİN TAŞIYICI/ADJUVAN ÖZELLİKLERİ KULLANILARAK YENİ AŞI TEKNOLOJİSİ GELİŞTİRİLMESİ

Patojen ile ilişkili moleküler yapılar (PAMP'ler) ve tehlike ile ilişkili moleküler yapılar (DAMP'ler), doğuştan gelen immün yanıtların düzenlenmesi için sitozol içindeki NLR reseptör ailesi tarafından algılanır. NLRP3 gibi bazı NLR'ler, enflamasyon komplekslerin oluşumunu indükler. Kaspaz-1, apoptozla ilişkili zerrecik benzeri bir protein (ASC) ile etkileşime girerek inflamazom multiprotein kompleksi içinde aktive edilir. Apoptoz ile ilişkili zerrecik benzeri CARD içeren protein (ASC) 22 kDa büyüklüğünde, bir C-terminali kaspaz alım alanı (CARD) ve bir N-terminal pirin (PYD) alanı içeren sitozolik proteindir. Uyarıldıktan sonra, ASC filamentleri, birçok ASC molekülü ile birleşerek ASC zerreciği adı verilen küresel bir yapı oluşturur. Bu zerreler hücrenin içinde bulunabilir ve ya birçok sitokin gibi sinyalleri yükseltmek için hücre dışı boşluğa salınabilir. Grubumuzun önceki verilerinde ASC zerrelerine yapay yükleme yapıldığı gösterildi. PBS içerisindeki ASC zerrelerinin 37 ° C'de 30 gün boyunca stabil kaldığını gösterdik. Ayrıca ASC zerrelerinin farelerin dalağında uzun süre kalıcı olarak kaldığını gösterdik. Tezin amacı, antijen yüklü ASC zerrelerinin saflaştırılması ve ASC zerrelerinin kullanılması ile aşılama yapılmasıdır. ASC zerrelerinin adjuvan özelliklerini ortaya koymaya yönelik, THP-1 makrofajlarının, saflaştırılmış ASC zerreleri ile uyarılmasından sonra, IL-1beta ve TNF-alpha gibi bazı enflamatuar sitokinlerin salgılanmasında artış olduğunu gösterdik. Ayrıca, kH5-ASC ile immünize edilmiş C57BL/6 fareleri, diğer immünize edilmiş fare gruplarına kıyasla daha yüksek anti-H5 IgG seviyelerine sahipti. Son olarak, i.p. enjekte edilen kOVA-ASC zerreleri, C57BL/6 farelerinde EG7-OVA timoma tümörüne gittiğini ve tümörde tam eradikasyon ve ya küçülmeye neden olduğunu gösterdik.

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## LIST OF SYMBOLS

|             |                        |
|-------------|------------------------|
| <i>bp</i>   | Base Pairs             |
| <i>g</i>    | Gravity                |
| <i>gr</i>   | Gram                   |
| <i>kDa</i>  | Kilodalton             |
| <i>L</i>    | Liter                  |
| <i>M</i>    | Molar                  |
| <i>mg</i>   | Milligram              |
| <i>mm</i>   | Millimeter             |
| <i>mM</i>   | Millimolar             |
| <i>min</i>  | Minute                 |
| <i>ng</i>   | Nanogram               |
| <i>rpm</i>  | Revolutions per Minute |
| <i>sec</i>  | Second                 |
| <i>V</i>    | Volt                   |
|             |                        |
| $^{\circ}C$ | Celcius Degree         |
| $\alpha$    | Alpha                  |
| $\beta$     | Beta                   |
| $\gamma$    | Gamma                  |
| $\mu g$     | Microgram              |
| $\mu l$     | Microliter             |
| $\mu m$     | Micrometer             |

## LIST OF ACRONYMS/ABBREVIATIONS

|                    |                                                           |
|--------------------|-----------------------------------------------------------|
| Ab                 | Antibody                                                  |
| AIM2               | Absent in Melanoma 2                                      |
| APC                | Antigen Presenting Cell                                   |
| APS                | Ammonium Persulfate                                       |
| ASC                | Apoptosis-Associated Speck-Like Protein Containing a CARD |
| BSA                | Bovine Serum Albumin                                      |
| CARD               | Caspase Recruitment Domain                                |
| cDNA               | Coplementary DNA                                          |
| C-terminal         | Carboxyl Terminal                                         |
| CytOVA             | Cytoplamic Ovalbumin                                      |
| DAMP               | Danger-associated Molecular Patterns                      |
| DAPI               | 4',6-diamidino-2-phenylindole                             |
| DC                 | Dendritic Cell                                            |
| ddH <sub>2</sub> O | Double Distilled Water                                    |
| dH <sub>2</sub> O  | Distilled Water                                           |
| DMEM               | Dulbecco's Modified Eagle Medium                          |
| DMSO               | Dimethyl Sulfoxide                                        |
| DNA                | Deoxyribonucleic Acid                                     |
| dNTP               | Four Deoxyribonucleotides                                 |
| dsDNA              | Double Stranded DNA                                       |
| EB                 | Elution Buffer                                            |
| eg.                | For example                                               |
| eGFP               | Enhanced Green Fluorescent Protein                        |
| ELISA              | Enzyme Linked Immunosorbent Assay                         |
| EtBr               | Ethidium Bromide                                          |
| FBS                | Fetal Bovine Serum                                        |
| H                  | Hemagglutinin                                             |
| hASC               | Human ASC                                                 |

|           |                                           |
|-----------|-------------------------------------------|
| HBS       | HEPES Balanced Salt                       |
| HEK       | Human Embryonic Kidney                    |
| HRP       | Horseradish Peroxidase                    |
| IL        | Interleukin                               |
| LB        | Luria-Bertani Broth                       |
| LPS       | Lipopolysaccharide                        |
| LRR       | Leucin Rich Repeat                        |
| MHC       | Major Histocompatibility complex          |
| mAb       | Monoclonal Antibody                       |
| mRNA      | Messenger RNA                             |
| N         | Neuraminidase                             |
| NEAA      | Non-essential Amino Acids                 |
| NF-KB     | Nuclear Factor Kappa B                    |
| NLR       | NOD-like Receptor                         |
| NLRP3     | NLR family, Pyrin Domain Containing 3     |
| NOD       | Nucleotide-binding Oligomerization Domain |
| OD        | Optical Density                           |
| OVA       | Chicken Ovalbumin Protein                 |
| PAMP      | Pathogen-associated Molecular Pattern     |
| PBS       | Phosphate Buffer Saline                   |
| PBS-T     | Phosphate Buffer Saline and Tween 20      |
| PCR       | Polymerase Chain Reaction                 |
| Pen/Strep | Penicillin/Streptomycin                   |
| PFA       | Paraform Aldehyde                         |
| PMA       | Phorbol Myristate Acetate                 |
| PRR       | Pathogen Recognition Receptor             |
| RBC       | Red Blood Cells                           |
| RT        | Room Temperature                          |
| SDS       | Sodium Dodecyl Sulfate                    |
| SDS-PAGE  | SDS Polyacrylamide Gel Electrophoresis    |
| TAE       | Tris Acetate EDTA                         |

|          |                                         |
|----------|-----------------------------------------|
| TBS      | Tris Buffered Saline                    |
| TBS-T    | Tris Buffered Saline and Tween 20       |
| TEMED    | N,N,N',N'-Tetramethylethane-1,2 diamine |
| TLR      | Toll Like Receptor                      |
| Tris     | Tris(hydroxymethyl) Aminomethane        |
| TNF      | Tumor Necrosis Factor                   |
| Tween 20 | Polysorbate                             |
| UV       | Ultraviolet                             |
| V        | Volume                                  |
| w        | Weight                                  |
| WB       | Western Blot                            |
| WT       | Wild Type                               |

# 1. INTRODUCTION

## 1.1. Immune System

Fight against microbes is mediated by the immune system. Innate immunity which is also called as native or natural immunity, provides the early reactions of defense against pathogenic microbes such as fungi, bacteria, viruses and parasites [1]. Innate immunity consists of biochemical and cellular defense mechanisms which are available in the local tissues before infection. These mechanisms provide an early and rapid responses to infections. Adaptive immunity, also called an acquired immunity, recognizes and distinguishes different substances with high specificity. It responds more vigorously to the same microbe in repeated exposures because it has memory. So, the activation of adaptive immunity is actually mediated by proper reactions of the innate immune responses [5].

### 1.1.1. Innate Immunity

Pathogen-associated molecular patterns (PAMPs) are evolutionarily conserved patterns on pathogens such as endotoxins on the bacterial cell membrane and bacterial lipopolysaccharides (LPS). Danger-associated molecular patterns (DAMPs) are cytosolic or nuclear proteins, or even ATP or DNA. PAMPs and DAMPs are detected by membrane-bound pathogen recognition receptors (PRRs) including Toll-like receptors (TLRs) or sensed by nucleotide binding oligomerization domain-like receptors (NLRs) in the cytosol in order to regulate the immediate responses of innate immunity [6].

### 1.1.2. Activation of Inflammasome

There are two major signals that regulate the formation of NLRP3 inflammasome. The first signaling known as priming is mediated by PRRs including C-type lectin receptors (CLRs) and TLRs upon binding to their respective PAMP or DAMP. This induces the NF - kappa B dependent transcription of NLRP3 and pro-IL-1beta. The

second signal is required for inflammasome complex formation leading to the activation of procaspase-1 [5].

NLRP3 dependent inflammasome formation requires the homotypic interaction between the PYD domains of NLRP3 and ASC and interaction between the CARD domains of ASC and procaspase-1. Thus activated caspase-1 cleaves pro-IL-1 $\beta$  and pro-IL-18 to their mature forms and these cytokines are released to the extracellular space to induce inflammation [7].

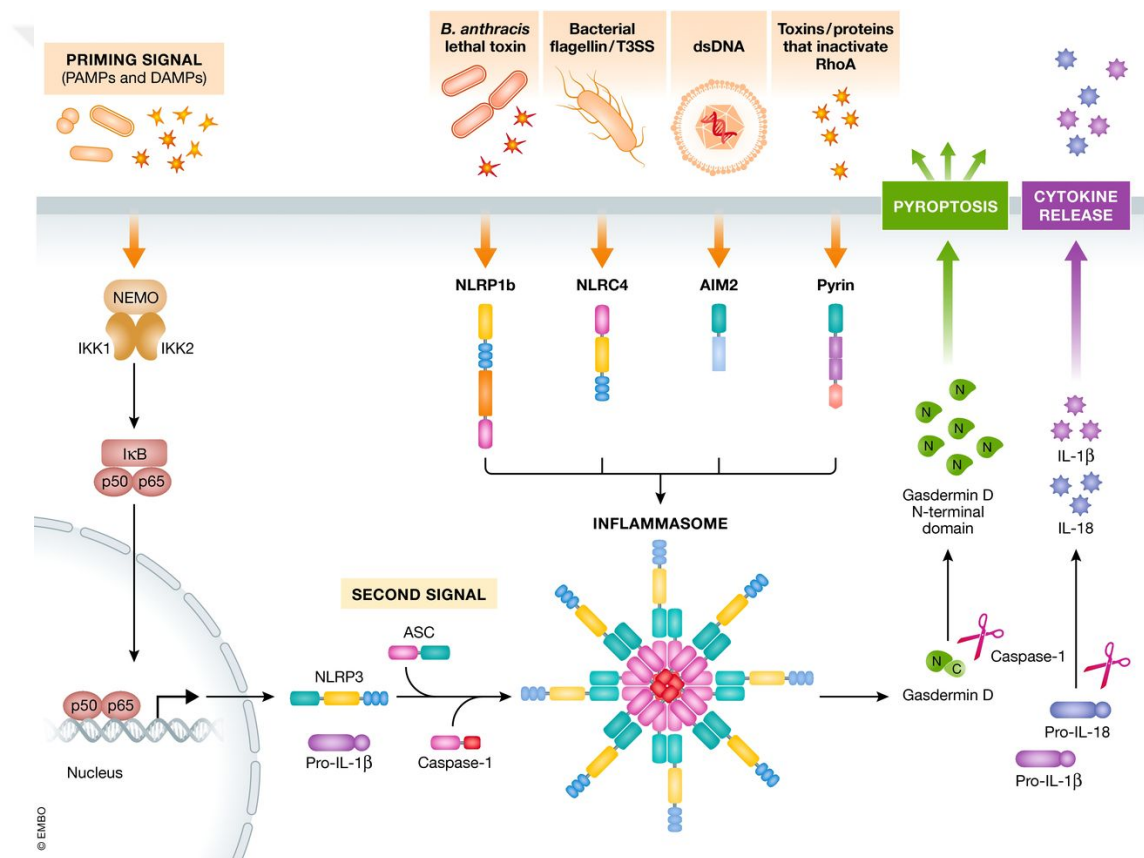


Figure 1.1. Inflammasome activation (Adopted from Voet, 2019.) [1]

### 1.1.3. ASC Speck Formation

The adaptor protein ASC is important for the activation of caspase-1 in response to many pathogens and disease associated signals. ASC protein is 22 kDa protein

found in the cytosol and contains C terminal CARD domain and N terminal PYD domain [8]. ASC has roles in both innate and adaptive immunity, such as modulation of the inflammasome, cell death or survival and antigen uptake and presentation [9].

ASC proteins polymerize into filaments and combine which compact further to oligomerize into a large supramolecular scaffold formation called as ASC speck [10]. These ASC specks can exist inside of the cell or can be released to the outside [11]. An ASC speck uptaken by macrophages can amplify the signals like many cytokines [12].

#### **1.1.4. Adaptive Immunity**

Adaptive immunity known as acquired immunity is a highly system for the recognition of pathogens to eliminate them. Following innate immunity, adaptive immunity is the second system to fight against microbes. Immunological memory is acquired after the proper activities of innate immunity and adaptive immunity [13]. Vaccination is based on this strategy, where antigens are used to induce the response of adaptive immunity [5].

T cell receptors (TCRs) are present on T lymphocytes and immunoglobulins (Ig) are expressed by B lymphocytes to detect the non-self agents for the adaptive immune response. Immunoglobulins bind to the free molecules (antigens) but TCRs can bind only to degraded antigen particles which are presented on MHC proteins. The huge variation of MHC molecules is provided by allelic polymorphisms. Junctional diversity, VDJ recombination and gene rearrangement are performed by recombinase activating gene (RAG) and the terminal deoxynucleotidyl transferase (TdT) to provide a diversity of TCRs and immunoglobulins. [13]

### **1.2. Antigen Presenting Pathways**

In order to fight against microbes, both innate immunity and adaptive immunity work together. T lymphocytes are activated via antigen presentation from APCs. MHC complexes on APCs provide the antigen presentation to T lymphocytes [2]. Triggered

innate immunity by PRRs improves the antigen presentation process. During vaccination, adjuvants are generally used to increase antibody production. Alum(Aluminium hydroxide) is a known adjuvant that stimulates the NLRP3 inflammasome in an ASC dependent manner.

### **1.2.1. Antigen Presentation by MHC Class I and MHC Class II**

MHC proteins including class I and II are expressed on APCs although all other nucleated express only MHC class I proteins. T lymphocytes recognize antigens on MHC class proteins via T cell antigen receptors (TCRs). CD8+ T cells recognize antigens on MHC class I while CD4+ T cells recognize antigens on MHC class II [14].

Antigens are engulfed by APCs and generally degraded by proteases and then loaded onto MHC class molecules de novo. Classical studies on antigen presentation showed that proteins synthesized inside the cells are presented on MHC class I molecules while exogenous proteins from the extracellular space are presented on MHC class II molecules. However, more studies showed that dendritic cells have a special capacity for processing of exogenous antigens to MHC class I pathway. This phenomenon is known as "cross-presentation" and provides the generation of an important mechanism in the immune system against viruses and self-antigens [15].

## **1.3. Vaccination**

Vaccines are defined as biological reagents that provide improvement of defense against infections according to the definition of the World Health Organization (WHO). Ingredients of vaccines are detected by innate immunity as "infection". These ingredients are engulfed by APCs which will engage the further response of adaptive immunity [16].

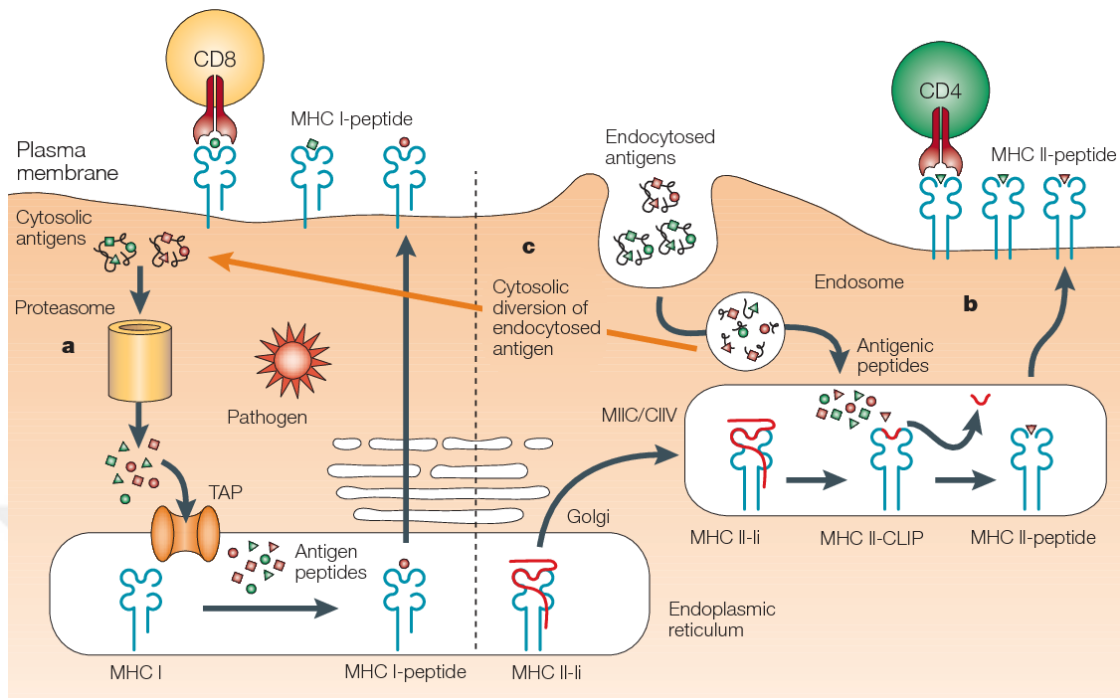


Figure 1.2. Antigen Presentation on MHC Class Proteins(Adopted from Heath, 2001.) [2]

### 1.3.1. History of Vaccines

In the 18th century, smallpox disease was an epidemic. Edward Jenner developed a precursor treatment to cure the disease. Using small lesions of cowpox to inoculate healthy people, he showed that the death rate was significantly decreased for smallpox. This method is actually known as vaccination [17]. After the discovery of vaccination, scientists developed vaccines against many deadly diseases such as rubies and anthrax.

### 1.3.2. Importance of Vaccines

A majority of human deaths are a consequence of infectious diseases, especially in low-income countries. This circumstance shows a relation between the economy and health. This unequal situation can be removed by vaccination because it reduces health care costs through controlling and curing deadly diseases. Thanks to vaccination, mortality and morbidity rates for children decreased dramatically over the last 50

years. This provided an improvement of the economic and social lives of families. Besides, vaccination increased the average life-span [18].

### 1.3.3. Influenza Virus

There are several types of influenza viruses including A, B and C. These different types affect different organisms and their basic differences are based on hemagglutinin and neuraminidase proteins [19]. Type A flu viruses can infect both animals and humans. Especially birds are common hosts for this type of viruses. This virus type is constitutively changing and most of the large flu epidemics are the consequence of this virus type. The characteristics of type A virus is defined as severe, extremely harmful and epidemic. Type B viruses are only found in humans. The severity of type B viruses is less severe than type A viruses. Thus type B does not cause pandemics. Type C flu viruses are also found in humans but they have milder effects than type A and B. Type C does not cause epidemics [20].

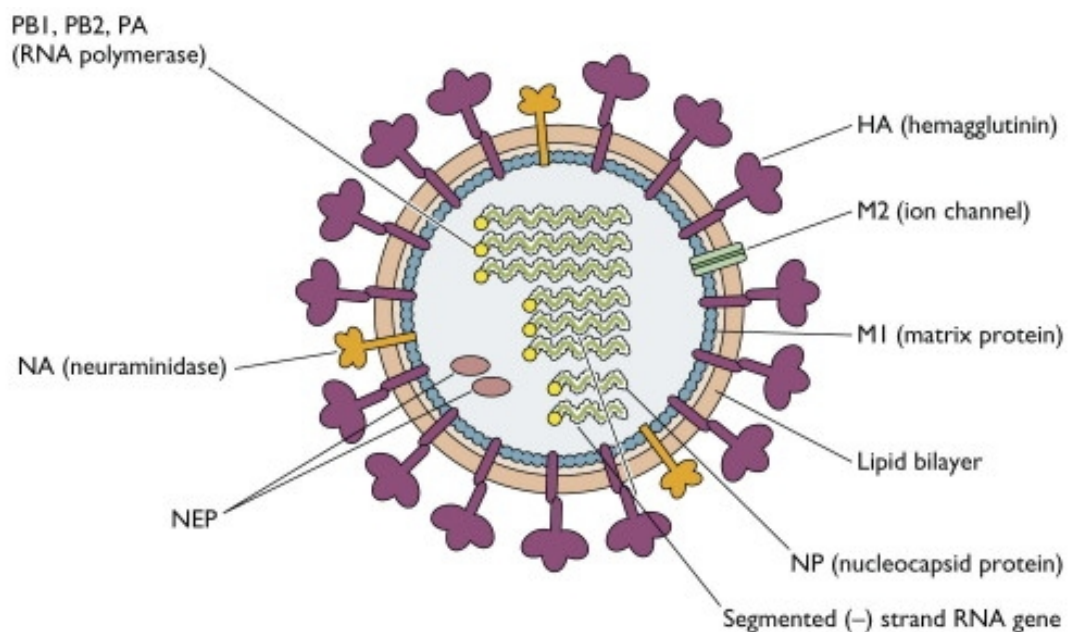


Figure 1.3. Structure of an influenza virus.(Adopted from [www.virology.ws](http://www.virology.ws))

1.3.3.1. Influenza A Virus H5N1. H5N1 is also known as avian influenza and belongs to type A viruses. Strains of avian influenza are divided into subtypes based on pathogenicity including low pathogenicity or high pathogenicity. In 1996, the highly pathogenic strain of H5N1 appeared in China. H5N1 is a global concern as a pandemic threat. Millions of people in different countries such as Asia, Europe and Africa have been killed by H5N1. There is a general concern about the coexistence of avian flu viruses and human flu viruses which can exchange their genetic material to create a new virulent form of influenza strain that has an easy transmissible property and is lethal to humans [21].

1.3.3.2. Hemagglutinin. Hemagglutinin (HA) are glycoproteins that are contained on the surface of viruses as coat proteins. These proteins provide the binding of the virus to the sialic acids present on certain receptors on the host cell membrane. Influenza viruses commonly have an effect on the upper respiratory tract because epithelial cells in those parts have sialic acid. The viral envelope is fused with the endosomal membrane via hemagglutinins. There are several HA antigens named as H1, H3, H5 and so on. A single change in the amino acid sequence of HA can lead to receptor binding ability to infect human cells [22].

#### **1.3.4. Types of Vaccines**

The most effective way to infections or severe outcomes from influenza viruses is vaccination. Scientists use many approaches to design vaccines against microbes. These approaches are generally based on information about microbes such as how they infect the cells and how the immunity responds to the infection [23].

1.3.4.1. Live-attenuated Vaccines. Live-attenuated vaccines contain a different version of living microbes that have been weakened in the laboratory thus they cannot cause disease. These vaccines are the closest version to natural infections so they are good informative vaccines for the immune system. There is a nature of living things to change. So, these live-attenuated vaccines can mutate and revert to virulent form

to cause disease. Besides, people with have weakened or damaged immune systems cannot take these vaccines safely. Also, these vaccines need cold temperatures to stay stable and potent. So, transportation of these vaccines to overseas or remote areas and storage of the vaccines for a long term may not easy [24].

1.3.4.2. Inactivated Vaccines. Inactivated vaccines are produced by killing the disease-causing microbe with heat, chemicals or radiation in the laboratory. These vaccines are safer and more stable than live-attenuated vaccines because dead microbes can not mutate back to the disease-causing state. Also, inactivated vaccines do not need refrigeration to stay stable and potent. They can be stored and transported easily. However, these vaccines stimulate the immune system weakly compared to live vaccines so, these vaccines are generally given in several doses to achieve stable immunity. It might not be possible to take booster shots on time for some remote areas, where people do not have access to health care regularly [24].

1.3.4.3. Subunit Vaccines. Subunit vaccines contain only antigens that determined to be the best stimulant for that particular pathogen. These vaccines include epitopes that are the specific parts of an antigen that are recognized by APCs. The unfavorable reactions to this kind of vaccines are lower because they include only very specific antigens, and not other molecules. From the 1 to more antigens can be included in these vaccines but the identification of the antigens that are best to stimulate and achieve protective immunity is a time-consuming and tricky process [24].

## **1.4. Antigen Delivery Systems and Adjuvants**

The efficiency of vaccines can be increased by using adjuvants to boost the innate immune reactions and induce the adaptive immunity more efficiently. Mineral salts, microbial particles, liposomes and emulsions can be included in the vaccines as adjuvants [25]. Aluminum salts (Alum) are commonly used adjuvants in human vaccines. A few recently published research articles indicate that Alum induces the immune system via caspase-1 and IL-1 beta and 18, which are the products of inflammasome

complex [26].

#### 1.4.1. Adjuvant Immunology

A number of action mechanisms have been elucidated about the effects of adjuvants in immune signalling. Many adjuvants might act as ligands for PRRs to activate innate immunity. Signaling from receptors then can activate certain transcription factors to induce expression of chemokines and cytokines that help to boost the direct immune response, such as TH1 or TH2 type response as well as effect immune cells that are recruited to the site of infection. Activation of inflammasome has also been shown as an active mechanism for some of the adjuvants. Inflammasome activation induces the production of pro-inflammatory cytokines IL-1 $\beta$  and IL-18. Also, certain adjuvants influence the presentation of antigens by MHC class proteins. It is also possible that some adjuvants can have multiple action mechanisms; for instance, it has been proposed that Alum can affect PRR signaling, antigen uptake, recruitment of the immune cells to the site of injection and activation of the inflammasome [27].

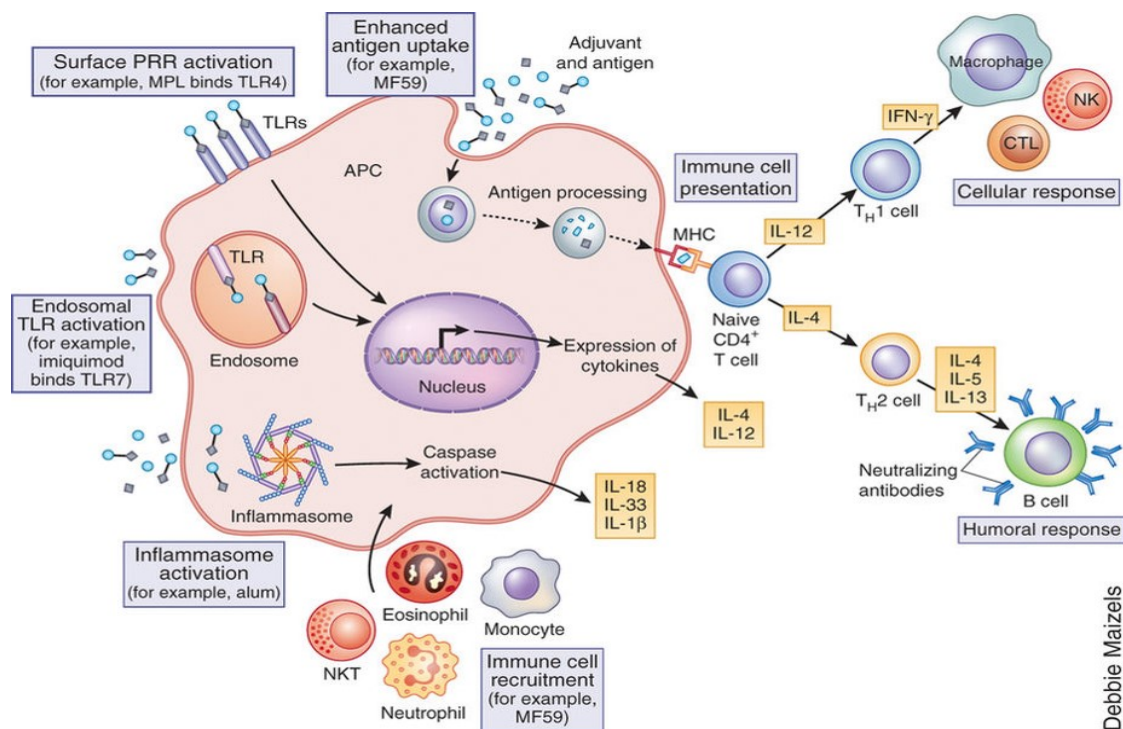


Figure 1.4. Action Mechanisms of Adjuvants (Adopted from Rambe, 2015.) [3]

### 1.5. Cancer Immunotherapy

Recent studies in basic immunology and tumor biology provide new insights into the understanding of the interaction between the immune system and tumor cells. Indeed, many studies have established the importance of T cells to mediate anti-tumor immunity. The process of carcinogenesis leads to many changes in the expression of antigens for tumor cells, which can be recognized by host T cells [28]. Currently, immune checkpoint inhibitors engineered T cells, monoclonal antibodies and cancer vaccines are being commonly used as immunotherapy against many types of cancers [29]. The anti-tumor monoclonal antibody binds to a specific antigen on the tumor cell and connects an Fc receptor on the killer cell. This situation induces direct cytotoxicity or antibody promoted phagocytosis, resulting in processing and presentation of antigens via MHC Class I or II molecules on APCs. Thus, it induces the host anti-tumor immunity revealed by either producing tumor-directed host antibodies or cytotoxic T-lymphocytes [4]

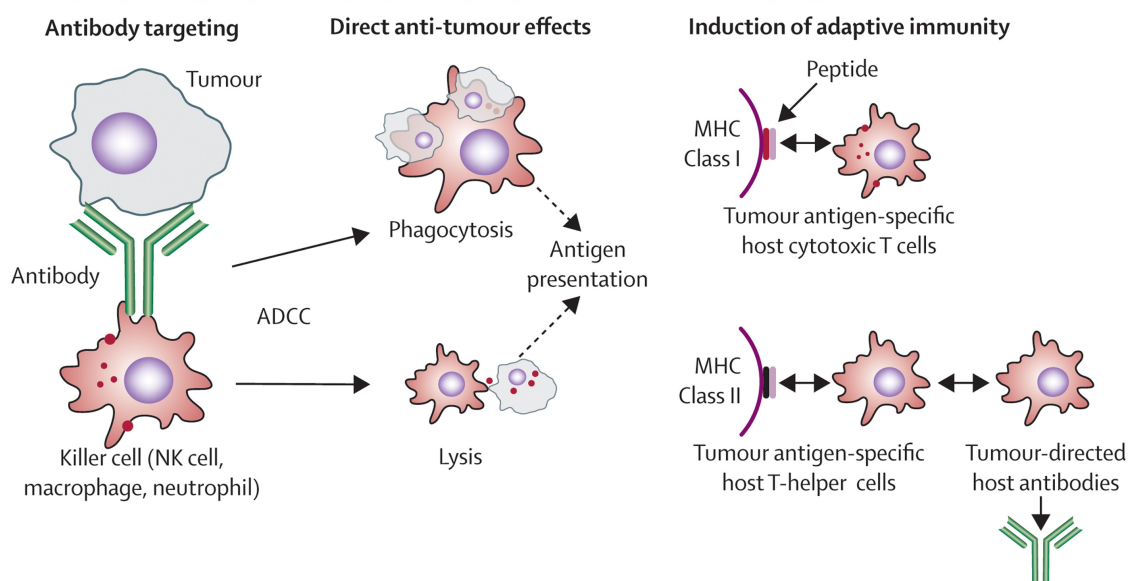


Figure 1.5. Monoclonal antibody mediated cancer immunotherapy (Adopted from Weiner, 2009.) [4]

### 1.5.1. Cancer Vaccines

Innovative approaches have been developed after the identification of a certain "signature" antigen on human tumor cell resulting in the development of antigen-specific vaccination. Combination of certain tumor antigens and proper routes for the delivery of these antigens to the immune system aims for optimizing human-based vaccination [28].

1.5.1.1. Tumour cell-based vaccines. Tumor cells can be used as vaccines for people with cancer. These tumor cells are not able to cause an immune response adequately in patients, they led to attempt to overcome this unresponsiveness by introducing of co-stimulatory molecules or cytokine genes into the genome of tumor cells, that are used as vaccines and given back to the same patient. To perform this, micro-environmental conditions should be created to prevent anergy and for allowing tumor destruction. However, this kind of vaccine production is rather slow, labor-intensive and difficult in a logistic manner [28].

1.5.1.2. Peptide-based vaccines. In-vivo loading of MHC molecules on APCs that express co-stimulatory molecules is the basis of effective peptide-based vaccination. These vaccines can contain several T cell epitopes that cover a broad range of MHCs. This method can be successful for the induction of T cell responses that are polyclonal [28].

1.5.1.3. Recombinant virus-based vaccines. Recombinant viral vectors can be used for the delivery of tumor antigen as vaccines. The intrinsic ability of viral vectors for the initiation of immune responses and occurring inflammatory reactions as a result of viral infection are the advantages of these vaccines. However, a viral vector ideally should be safe and should not promote anti-vector immune responses to allow the boosting of anti-tumor specific immune responses [28].

1.5.1.4. DNA-based vaccines. DNA-based vaccines use DNA that encodes a specific tumor antigen and these vaccines are given to the patients directly. So, simplicity and low-cost production are the advantages of this type of vaccine. These vaccines can induce both T cell-mediated and humoral immunity if the whole antigen is delivered. DNA vaccines that encode weak immunogenic antigens could be improved by immunization with DNA that encodes adjuvant-like elements such as the constant region of an Ig or human cytokine GM-CSF (Granulocyte-macrophage colony-stimulating factor) to increase the efficiency of DNA-based vaccines [28].

1.5.1.5. Dendritic cells vaccines. Loading dendritic cells with tumor antigens or transfection of DCs with tumor antigen expressed viral vector or fusion of DCs with whole tumor cells are the strategies that have been used to develop dendritic cells vaccines. Immunization with DCs vaccines can provide rejection of existent tumor and generation of functional DCs can be performed in vitro so it can be attractive vaccination [28].

## 2. PURPOSE

For this project, there were three main purposes including determination of an adjuvant characteristic of ASC specks, development of a novel vaccine against H5N1 influenza and novel cancer immunotherapy using ASC specks. The first aim of the project is the loading of antigen that is the most antigenic part of the H5 coat protein of H5 influenza on ASC specks. The plasmid expressing tH5(69-133aa)-ASC was constructed for this project and after transfection of this plasmid into HEK293FT cell line, successful purification of tH5-ASC specks was performed. These ASC specks were used for the immunization of wild type(C57BL/6) mice against H5 coat protein of influenza. The antigen-specific IgG response was studied to see the effect of ASC specks compared to other experimental groups.

The second aim of this project is to elucidate the possible roles of the ASC specks as a novel adjuvant. Purified ASC specks were used in animal studies using C57BL/6 mice with a group including known adjuvant Alum to compare the adjuvant efficiency of ASC specks with Alum.

The last aim of this project is the development of novel cancer immunotherapy using ASC specks. To observe the effect of ASC specks on tumor progression, EG7-OVA tumor cells were inoculated on the right dorsal flank of C57BL/6 mice and after the tumor formations, tOVA-ASC specks were injected intraperitoneally to mice to observe the anti-tumor activity of tOVA-ASC specks such as a decrease in tumor size.

### 3. MATERIALS

#### 3.1. Cell Lines

Table 3.1. Cell lines used in this study.

|                   | Catalog No   | Main Source     | Provider                                         |
|-------------------|--------------|-----------------|--------------------------------------------------|
| HEK293(F)T        | R700-07      | Invitrogen, USA | Kindly provided by<br>Prof. Maria Soengas        |
| THP-1             | ATCC TIB-202 | ATTC, USA       | Kindly provided by<br>Prof. Ahmet Gül, Turkey    |
| THP-1<br>eGFP-ASC | -            | THP-1 Cell Line | Generated at<br>AKİL Lab, Turkey                 |
| EG7-OVA           | CRL-2113     | ATCC, USA       | Kindly provided by<br>Prof. İhsan Gürsel, Turkey |

#### 3.2. Buffers and Solutions

Table 3.2. Commercial buffers used in cell culture.

|                         | Catalog Number | Source                        |
|-------------------------|----------------|-------------------------------|
| 2-mercaptoethanol       | 21985-023      | Gibco, Life Technologies, USA |
| DMEM                    | 41966029       | Gibco, Thermo Fisher, USA     |
| DMSO                    | A3672          | AppliChem, Germany            |
| Fetal Bovine Serum      | 10270106       | Gibco, Thermo Fisher, USA     |
| G418 (Geneticin)        | 1150MG250      | Neofroxx, Germany             |
| MEM-NEAA                | 11140050       | Gibco, Thermo Fisher, USA     |
| Penicillin Streptomycin | 15140122       | Gibco, Thermo Fisher, USA     |
| RPMI Medium 1640        | 21875034       | Gibco, Thermo Fisher, USA     |

Table 3.3. Prepared buffers used in cell culture.

|                      |                                                                                                                                                                                                                                                                                                       |
|----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 0.05% Trypsin        | 0,53 mM EDTA (0.2 g EDTA.4Na)<br>0,5 g Trypsin<br>8 g NaCl<br>0,4 g KCl<br>0,06 g KH <sub>2</sub> PO <sub>4</sub><br>1 g D-Glucose<br>0,048 g Na <sub>2</sub> HPO <sub>4</sub><br>0,35 g NaHCO <sub>3</sub><br>In 1 Liter dH <sub>2</sub> O<br>Adjusted pH: 8.00<br>Sterilized by 0.22 $\mu$ m filter |
| 10X PBS              | 80 g NaCl<br>2 g KCl<br>2.4 g KH <sub>2</sub> PO <sub>4</sub><br>14.4 g Na <sub>2</sub> HPO <sub>4</sub><br>In 1 L dH <sub>2</sub> O<br>Adjusted pH: 7.4                                                                                                                                              |
| 2M CaCl <sub>2</sub> | 88 g CaCl <sub>2</sub> in 500 ml dH <sub>2</sub> O<br>Sterilized by 0.22 $\mu$ m filter                                                                                                                                                                                                               |
| 2X HBS               | 4.08 g NaCl<br>0.18 g KCl<br>0.053 g Na <sub>2</sub> HPO <sub>4</sub><br>0.59 g D-Glucose<br>2.97 g HEPES<br>In 200 ml dH <sub>2</sub> O<br>Adjusted pH: 7.05<br>Sterilized by 0.22 $\mu$ m filter                                                                                                    |

Table 3.3. Prepared buffers used in cell culture. (cont.)

|                  |                                                                                      |
|------------------|--------------------------------------------------------------------------------------|
| ACK Lysis Buffer | 154 mM NaH <sub>4</sub> Cl<br>10 mM KHCO <sub>3</sub><br>0.1 mM Na <sub>2</sub> EDTA |
| Complete DMEM    | 500 ml DMEM<br>50 ml FBS-HI<br>5.5 ml Pen-Strep<br>5.5 ml MEM NEAA                   |
| Freezing Medium  | 10% FBS<br>1X Pen-Strep<br>100 µM MEM NEAA<br>10% DMSO<br>In Growth Medium           |

Table 3.4. Western blot solutions.

|                                 |                                                                                                                        |
|---------------------------------|------------------------------------------------------------------------------------------------------------------------|
| 30:0,8 Acrylamide-Bisacrylamide | 30 g Acrylamide<br>0,8 g Bisacrylamide<br>In 100 ml dH <sub>2</sub> O                                                  |
| 4% Stacking Gel                 | 66 ml 30:0,8 Acrylamide-Bisacrylamide<br>126 ml 0,5 M Tris-HCl(pH:6,8)<br>5 ml SDS<br>In 500 ml dH <sub>2</sub> O      |
| 15% Resolving Gel               | 500 ml 30:0,8 Acrylamide-Bisacrylamide<br>200 ml 1,875 M Tris-HCl(pH:8,8)<br>10 ml SDS<br>In 1 Liter dH <sub>2</sub> O |

Table 3.4. Western blot solutions. (cont.)

|                                   |                                                                                                                                           |
|-----------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------|
| 5X Laemmli Buffer                 | 1,5M Tris-HCl(pH:6,8)<br>10 ml Glycerol<br>5 ml beta-mercaptoethanol<br>2 g SDS<br>1 ml 1% bromophenol blue<br>In 20 ml dH <sub>2</sub> O |
| APS                               | AppliChem, Germany                                                                                                                        |
| TEMED                             | Sigma, Germany                                                                                                                            |
| Tween-20                          | Sigma, Germany                                                                                                                            |
| 10X Tris-Glycine (Running) Buffer | 30 g Tris base<br>144 g Glycine<br>In 1 Liter dH <sub>2</sub> O                                                                           |
| Semi-dry Transfer Buffer          | 2,93 g Glycine (39 mM)<br>5,81 g Tris-base (47 mM)<br>6,25 ml 10% SDS<br>In 1 Liter dH <sub>2</sub> O<br>Adjusted pH: 9.2                 |
| Methanol                          | Merck, USA                                                                                                                                |
| 0.5 M Tris-HCl (pH:6.8)           | 6.06 g Tris base<br>In 100 ml dH <sub>2</sub> O<br>Adjust pH to 6.8 with HCl                                                              |
| 1.5 M Tris-HCl (pH:8.8)           | 78.5 g Tris base<br>In 500 ml dH <sub>2</sub> O<br>Adjust pH to 8.8 with HCl                                                              |
| 10X TBS                           | 90 g NaCl<br>121,14 g Tris base<br>In 1 Liter dH <sub>2</sub> O                                                                           |

Table 3.4. Western blot solutions. (cont.)

|                                       |                                                                                |
|---------------------------------------|--------------------------------------------------------------------------------|
| TBS-T                                 | 100 ml 10X TBS<br>1 ml Tween-20<br>In 1 Liter dH <sub>2</sub> O                |
| Bovine Serum Albumin (BSA)            | Roche, Germany                                                                 |
| 5 % BSA                               | 5 g BSA<br>In 100 ml TBS-T                                                     |
| Sodium Azide                          | Roche, Germany                                                                 |
| Page Ruler Pre-stained Protein Ladder | Thermo Fischer Scientific, USA                                                 |
| ECL                                   | Advansta Inc., USA                                                             |
| Coomassie Blue Staining Solution      | 0.1% Coomassie Blue<br>10% Acetic acid<br>50% Methanol<br>In dH <sub>2</sub> O |
| Coomassie Blue Destaining Solution    | 10% Acetic acid<br>40% Methanol<br>In dH <sub>2</sub> O                        |
| Elution Buffer for SDS-PAGE           | 50 mM Tris-HCl<br>150 mM NaCl<br>0.1 mM EDTA<br>Adjusted pH: 7.5               |

Table 3.5. ELISA solutions.

|                    |                                                                                                               |
|--------------------|---------------------------------------------------------------------------------------------------------------|
| 1X PBS             | 100 ml 10X PBS<br>900 ml dH <sub>2</sub> O<br>Adjusted pH: 7.2-7.4<br>Filtered by 0.22 $\mu$ m filter         |
| PBS-T(Wash Buffer) | 0.05% Tween-20 in 1X PBS                                                                                      |
| Reagent Diluent    | 1% BSA in 1X PBS<br>Filtered by 0.22 $\mu$ m filter                                                           |
| Substrate Solution | 1:1 Mixture of Color Reagent A (H <sub>2</sub> O <sub>2</sub> )<br>and Color Reagent B (Tetramethylbenzidine) |
| Stop Solution      | 2N H <sub>2</sub> SO <sub>4</sub>                                                                             |

Table 3.6. Agarose Gel Electrophoresis.

|                                |                                                                                                                                  |
|--------------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| 10X TAE(Tris acedic acid EDTA) | 24,2 g Tris base<br>5,71 ml Glacial Acedic Acid<br>3,72 g Na <sub>2</sub> EDTA.2H <sub>2</sub> O<br>In 1 Liter dH <sub>2</sub> O |
| GeneRuler DNA Ladder Mix       | Thermo Fischer Scientific, USA                                                                                                   |
| Ethidium Bromide (EtBr)        | Merck,USA                                                                                                                        |
| 6X DNA Loading Dye             | Thermo Fischer Scientific, USA                                                                                                   |

Table 3.7. Enzymes and buffers used for molecular cloning.

|                                                         |              |
|---------------------------------------------------------|--------------|
| Taq Polymerase                                          | AKIL, TURKEY |
| Taq Polymerase Buffer                                   | AKIL, TURKEY |
| Q5 High Fidelity Polymerase                             | NEB, USA     |
| T4 DNA Ligase and T4 Ligase Buffer                      | NEB, USA     |
| Restriction Enzymes (NheI, HindIII, XhoI, EcorI, EcorV) | NEB, USA     |
| Alkaline Phosphatase (CIP)                              | NEB, USA     |

Table 3.8. Bacterial culture.

|                                         |                                                                                                                                                                                                    |
|-----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| LB Agar (Solid, autoclaved)             | 15 g Agar in 1 Liter ddH <sub>2</sub> O                                                                                                                                                            |
| LB Medium (Liquid, autoclaved)          | 10 g Tryptone<br>5 g Yeast Extract<br>5 g NaCl<br>In 1 Liter of dH <sub>2</sub> O                                                                                                                  |
| Modified LB Medium (Liquid, autoclaved) | 1X M9 Salt<br>0,2% Glucose<br>In LB medium                                                                                                                                                         |
| 10X M9 Salt                             | 70 g Na <sub>2</sub> HPO <sub>4</sub> ·7H <sub>2</sub> O<br>30 g KH <sub>2</sub> PO <sub>4</sub><br>5 g NaCl<br>10 g NH <sub>4</sub> Cl<br>In 1 Liter dH <sub>2</sub> O<br>Sterilized by autoclave |
| 20% Glucose (autoclaved)                | 20 g Glucose<br>In 100 mL dH <sub>2</sub> O                                                                                                                                                        |
| Ampicillin (1000X)                      | 0.5 g Kanamycin Sulfate<br>In 10 ml dH <sub>2</sub> O                                                                                                                                              |
| Kanamycin (1000x)                       | 0.5 g Kanamycin Sulfate<br>In 10 ml dH <sub>2</sub> O                                                                                                                                              |
| Chloramphenicol (1000x)                 | 0.34 g Chloramphenicol<br>In 10 ml Absolute Ethanol                                                                                                                                                |

### 3.3. Fine Chemicals

#### 3.3.1. Plasmids

Table 3.9. Plasmids used in this study.

| Name                   | Origin                     |
|------------------------|----------------------------|
| Pet30a H5              | Pet30a(+)AKIL, Turkey      |
| p-C3-eGFP-ASC          | pEGFP-C3, AKIL, Turkey     |
| p-C3-mCherry-ASC       | pEGFP-C3, AKIL, Turkey     |
| p-C3-tOVA(1-238aa)-ASC | pEGFP-C3, AKIL, Turkey     |
| p-C3-tH5(69-133aa)-ASC | Constructed by Aylin Alkan |
| Pet30a H1              | Constructed by Aylin Alkan |

#### 3.3.2. Primers

Table 3.10. Primers used in this study.

|                   |                                 |
|-------------------|---------------------------------|
| tH5-ASC-FP        | TACAAGGCTAGCATTATGAGAGATTGTAGTG |
| tH5-ASC-RP        | GACCAGAAGCTTGATGATCTGAATTTTCTC  |
| tH5-ASC-RP Seq    | TTGTCGGTGAGGTCCAAGG             |
| tH5ASC-ASC-M1-A-F | GATCCTGGAGCCGCGGGGCGCGCG        |
| tH5ASC-ASC-M1-A-R | CGCGCGCCCCGCGGCTCCAGGATC        |
| H1-ForwardP       | TCAGATATCATGAAGGCAATACTAGTAGTT  |
| H1-ReverseP       | ACTCTCGAGTTAAATACATATTCTACACTG  |
| H1-FP-Seq1        | TCCCCAAGACAAGTTC                |
| H1-FP-Seq2        | TCCCATTTCAGAATAT                |

### 3.3.3. Antibodies

Table 3.11. Antibodies used in this study.

|                              | Catalog Number | Provider                                    |
|------------------------------|----------------|---------------------------------------------|
| Anti-humanASC (mAb, mouse)   | -              | Kindly provided by<br>Prof. Masumoto, Japan |
| Anti-TMS1 (mAb, rabbit)      | ab155970       | Abcam, UK                                   |
| Anti-OVA (mAb, mouse)        | ab17293        | Abcam, UK                                   |
| Anti-H5 (mAb, mouse)         | MAB1136-M01    | Abnova, Taiwan                              |
| Anti-beta-actin (mAb, mouse) | 4970S          | Cell Signaling Technology,<br>Netherlands   |
| Anti-mouse IgG, HRP linked   | 7076S          | Cell Signaling Technology,<br>Netherlands   |
| Anti-rabbit IgG, HRP linked  | 7074S          | Cell Signaling Technology,<br>Netherlands   |

### 3.3.4. Injected Materials for Animal Experiments

Table 3.12. Injected materials for animal experiments.

|                                                        | Catalog No | Provider                                    |
|--------------------------------------------------------|------------|---------------------------------------------|
| ALUM(Aluminum Potassium<br>Sulfate Dodecahydrate)      | 237086     | Sigma, Germany                              |
| Chicken Ovalbumin<br>Low Endo <sup>TM</sup> , Purified | LS003059   | Worthington Biochemical<br>Corporation, USA |

### 3.4. Kits

Table 3.13. Kits used in this study.

|                                     | Catalog No | Provider                     |
|-------------------------------------|------------|------------------------------|
| NucleoBond® Xtra Midi EF            | 740420.10  | Macherey-Nagel, Germany      |
| NucleoSpin® Gel and<br>PCR Clean-up | 740609.50  | Macherey-Nagel, Germany      |
| NucleoSpin® Plasmid                 | 740588.50  | Macherey-Nagel, Germany      |
| Pierce™ BCA<br>Protein Assay Kit    | 23225      | ThermoFisher Scientific, USA |

### 3.5. Equipment

Table 3.14. Equipment used in this study.

|                               |                                                                                                                              |
|-------------------------------|------------------------------------------------------------------------------------------------------------------------------|
| Agarose Imaging               | Mini LED Transilluminator, IO Rodeo, USA                                                                                     |
| Autoclaves                    | AMA260BT, Astell Scientific, UK<br>ASB270N, Astell Scientific, UK                                                            |
| Centrifuges                   | Allegra X-22R, Beckman Coulter, USA<br>Himac CT15E, Hitachi Koki, Japan<br>J2-21, Beckman Coulter, USA<br>MiniStar, VWR, USA |
| Freezers                      | UFR370 SD, Uğur, Turkey<br>2020 ND, Arçelik, Turkey<br>TS 368, Thermo Fisher Scientific, USA                                 |
| Incubator                     | Forma Series II 3111, Thermo Fisher<br>Scientific, USA                                                                       |
| Heat Block                    | MyBlock Mini, Benchmark Scientific, USA                                                                                      |
| Horizontal<br>Electrophoresis | Mini gel electrophoresis kit, IO Rodeo, USA<br>MP-1015, IBI Scientific, USA                                                  |
| Laminar Flow Cabinet          | Class II A, Tezsan, Turkey                                                                                                   |
| Magnetic Stirrer              | Topolino, IKA, Germany                                                                                                       |

Table 3.14. Equipment used in this study. (cont.)

|                          |                                                                                                                                                               |
|--------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Microscopes              | Eclipse TS100, Nikon Corporation, Japan<br>TCS SP5 II, Leica Microsystems, Germany                                                                            |
| Microwave Oven           | MD-555 S, Arçelik, Turkey                                                                                                                                     |
| pH Meter                 | pH2700, Oakton Instruments, USA                                                                                                                               |
| Pipettes                 | Acura 826 Electro Series, Socorex, Switzerland<br>Axygen Axyjet Series, Corning, USA<br>Finnpipette F2, Thermo Fisher Scientific, USA<br>Accurpette, VWR, USA |
| Refrigerators            | USS 374 DTKY, Uğur, Turkey<br>S1150A, Vestel, Turkey                                                                                                          |
| Power Supplies           | Electrophoresis PSU Kit, IO Rodeo, USA<br>PowerPac Basic, Bio Rad, USA                                                                                        |
| Thermal Cyclers          | C1000, Bio Rad, USA<br>OpenPCR, Chai Biotechnologies, USA                                                                                                     |
| Thermal Shakers          | Forma 420, Thermo Fisher Scientific, USA<br>CTS-1, Inovia Teknoloji, Turkey                                                                                   |
| Shakers                  | PSU-10i, Grant, UK                                                                                                                                            |
| Spectrophotometer        | NanoDrop ND-100, Thermo Fisher Scientific, USA                                                                                                                |
| Vortex Shaker            | Vortexer, Heathrow Scientific, USA                                                                                                                            |
| Vertical Electrophoresis | Mini PROTEAN Tetracell, Bio-Rad, USA                                                                                                                          |
| Water Baths              | 1002, GFL, Germany<br>WB 14, Memmert, Germany                                                                                                                 |

## 4. METHODS

### 4.1. Molecular Cloning

#### 4.1.1. Isolation of Plasmid DNA

Isolation of plasmid DNA was performed according to instructions of the manufacturer (MN, NucleoSpin Plasmid, Germany). Briefly, 5 ml liquid culture of bacteria (including antibiotic(s)) was incubated on a shaker at 37°C for ON. Then, Bacteria culture was centrifuged at 11000g for 5 min. The supernatant was thrown away and the recommended amount of buffers were added and protocol for each step was performed according to instructions of the manufacturer respectively. Lastly, plasmid DNA was eluted with 50  $\mu$ l ddH<sub>2</sub>O from the mini-spin column. The yield of plasmid DNA and the ratios of 260/280 and 260/230 for plasmid DNA was calculated by using a spectrophotometer.

#### 4.1.2. High-Fidelity PCR Reaction

DNA fragments which are desired to be cloned into vector plasmids were amplified using Q5 High Fidelity DNA Polymerase (M0941, NEB, USA). PCR reactions were performed according to instructions of the manufacturer. Reaction components are summarized in Table 4.1. Briefly, recommended amounts of 5X Q5 reaction buffer, 10 mM dNTPs, forward and reverse primers, 5X Q5 High GC enhancer and nuclease-free water added in 1 microcentrifuge tube. If more than 1 sample was used, this mixture used as reaction mixture and shared equally into PCR tubes. Then DNA added into PCR tubes. Lastly, DNA polymerase was added into each tube and PCR reaction was started according to Table 4.2.

Table 4.1. Reaction components of PCR reaction for corresponding volume.

| Component                          | 25 $\mu$ l Reaction | 50 $\mu$ l Reaction | Final [C]       |
|------------------------------------|---------------------|---------------------|-----------------|
| 5X Q5 Reaction Buffer              | 5 $\mu$ l           | 10 $\mu$ l          | 1X              |
| 10 mM dNTPs                        | 0,5 $\mu$ l         | 1 $\mu$ l           | 200 $\mu$ M     |
| 10 $\mu$ M Forward Primer          | 1,25 $\mu$ l        | 2,5 $\mu$ l         | 0,5 $\mu$ M     |
| 10 $\mu$ M Reverse Primer          | 1,25 $\mu$ l        | 2,5 $\mu$ l         | 0,5 $\mu$ M     |
| Template DNA                       | variable            | variable            | $\leq 1000ng$   |
| Q5 High-Fidelity<br>DNA Polymerase | 0,25 $\mu$ l        | 0,50 $\mu$ l        | 0.02 U/ $\mu$ l |
| 5X Q5 High GC Enhancer             | 5 $\mu$ l           | 10 $\mu$ l          | 1X              |
| Nuclease Free Water                | Up to 25 $\mu$ l    | Up to 50 $\mu$ l    | -               |

Table 4.2. Thermo-cycling conditions for PCR reaction.

| Step                 | Temperature                    | Time       |
|----------------------|--------------------------------|------------|
| Initial denaturation | 98 °C                          | 30 seconds |
| (25-35 cycles)       |                                |            |
| Denaturation         | 98 °C                          | 10 seconds |
| Annealing            | Primer specific T <sub>m</sub> | 30 seconds |
| Elongation           | 72 °C                          | 30 seconds |
| Final extension      | 72 °C                          | 2 minutes  |
| Hold                 | 4 °C                           | $\infty$   |

#### 4.1.3. Agarose Gel Electrophoresis

Running of DNA fragments was performed on agarose gel for the procedure of agarose gel extraction and diagnostic purposes. Generally, 1% agarose gel was used. If DNA fragments were larger than 4 kb, 0.8% agarose gels were used. If DNA fragments were smaller than 200 bp, 1.5% agarose gels were used. For agarose gel preparation, 0,5 g agarose was weighted and re-suspended in 50 ml 1X TAE buffer. The solution was

boiled in the microwave until agarose was completely dissolved in the buffer. Then, the solution was incubated at RT for 5 minutes for the cooling process. 2,5  $\mu$ l EtBr was added under a fume hood, combs were put onto the gel after the gel became completely solid, it was put into agarose tank. DNA samples with DNA loading dye were run in 1X TAE buffer containing agarose gel electrophoresis tanks.

#### **4.1.4. Agarose Gel Extraction and PCR Purification after PCR Reaction**

PCR products were loaded into 1% agarose gel and run at 90 V for 30-40 minutes. Then, gel was placed onto UV light and was cut using a razor. The PCR products was extracted using the protocol of agarose gel extraction according to the instructions of manufacturer (MN, NucleoSpin Gel and PCR Clean-up, Germany).

#### **4.1.5. Restriction and Digestion of PCR Products and Vector Plasmids**

Vector plasmid DNAs and purified PCR products were digested with proper restriction enzymes. Restriction and digestion reactions were carried out according to instructions of the manufacturer (NEB, USA). A typical restriction digest protocol was shown in Table 4.3. For double digestion protocol, the reaction was restricted with the enzymes at different times. Firstly, one end was cut with one enzyme, then products were run into 1% agarose gel and agarose gel extraction and PCR purification methods were performed as mentioned above. After that, the other end was cut with the other enzyme, then products were run into 1% agarose gel and agarose gel extraction and PCR purification methods were performed. For this protocol, it is important that the volume of DNA should be less than  $\frac{1}{4}$  of the total reaction volume because the amount of salt concentration can inhibit the working of reaction properly. It is also important that if you are using 1 restriction enzyme, you have to add 1  $\mu$ l CIP (Calf Intestinal Alkaline Phosphatase) in the tube including vector plasmid in order to avoid self-ligation.

Table 4.3. Protocol for a typical restrict digestion experiment.

|                                 |                                                  |
|---------------------------------|--------------------------------------------------|
| Restriction Enzyme              | For 10 units 1 $\mu$ l, For 20 units 0,5 $\mu$ l |
| DNA                             | 1-5 $\mu$ g                                      |
| 10X NEBuffer                    | 5 $\mu$ l (1X)                                   |
| Total Reaction Volume           | 50 $\mu$ l                                       |
| Incubation Time and Temperature | 3 hours at 37 °C                                 |

#### 4.1.6. Ligation

Ligation of restricted DNA fragments and vector plasmids was done by using T4 polymerase (NEB, USA). In ligation reactions, amounts of the insert and vector plasmid were determined using online application tool according to the size of vector plasmid, size of insert DNA and the ratio of vector/insert. Ligation protocol was performed according to instructions of the manufacturer. For ligation protocol, it is important that the volume of DNA should be less than  $\frac{1}{4}$  of total reaction volume because the amount of salt concentration can inhibit the working of reaction properly. Besides, T4 DNA ligase buffer should be properly mixed to avoid salt precipitations in the buffer and T4 DNA ligase should be added last. A protocol of typical ligation was shown in Table 4.4. The ligation mix was incubated at 16 °C for 16 hours then, ligase was heat inactivated at 65 °C for 10 minutes. 5  $\mu$ l of the ligation mix was used for the transformation experiment.

Table 4.4. Typical ligation protocol for molecular cloning.

| Component                  | 20 $\mu$ l Ligation Reaction |
|----------------------------|------------------------------|
| T4 DNA Ligase Buffer (10X) | 2 $\mu$ l                    |
| Vector DNA                 | 50 ng                        |
| Insert DNA                 | Variable                     |
| Nuclease Free Water        | Up to 20 $\mu$ l             |
| T4 DNA Ligase              | 1 $\mu$ l                    |

#### 4.1.7. Transformation

The new constructs of plasmids were transformed into *E. coli* strains (Stbl3 or Rosetta). If the plasmid construct was to be used for only plasmid purification for further experiments, then Stbl3 competent cells were used. If the plasmid construct was to be induced with IPTG for protein purification, firstly Stbl3 competent cells were used, plasmids were isolated from them then the second transformation was performed using Rosetta strain. For ligation protocol, first competent cells were thawed on ice for 10-15 minutes. 5  $\mu$ l of ligation mixture (well mixed) was added to the microcentrifuge tube containing competent cells. DNA and competent cells were mixed gently by pipetting up and down. The mixture was placed on ice for 30 minutes. Then, heat shock was performed at 42 °C for 60-90 seconds. 950  $\mu$ l of LB media without antibiotics was added for each tube. They were shaken (200-250 rpm) at 37 °C for 60 minutes. Plates including antibiotics were warmed at RT during this time. The tubes were centrifuged at 11000g for 30 seconds. 800-900  $\mu$ l of media was thrown away. Remaining media including cells were spread onto the plates and they were incubated at 37 °C for ON.

#### 4.1.8. Preparation of Bacteria Stock

After incubation of bacterial culture for ON, at least 500  $\mu$ l of the overnight culture was taken into the sterile new micro-centrifuge tube or cryovial. 500  $\mu$ l of 50% glycerol was added and the mixture was gently mixed by pipetting up and down. The tubes were stored at -80 °C for long term storage. The final concentration of glycerol should be in 15-25%.

#### 4.1.9. Colony PCR

After ligation, positive colonies were screened with colony PCR experiment. Briefly, a pinch of bacteria colonies was taken and added into 10  $\mu$ l of ddH<sub>2</sub>O. These samples were incubated at 95 °C for 10 minutes. During this time, the PCR mixture was prepared according to Table 4.5. Primers for colony PCR ideally was chosen. One

of the primers anneals to the insert and the other anneal to the vector thus only the recombinant DNA is able to give the correct size of the band. Lastly, the mixture of PCR was added to each tube and colony PCR protocol was run according to Table 4.6. After PCR was done, samples were loaded into agarose gel to check positive colonies.

Table 4.5. Reaction components of colony PCR reaction for corresponding volume.

| Component                 | 1 Sample Rxn.    | 5 Samples Rxn.   |
|---------------------------|------------------|------------------|
| 10X Taq Reaction Buffer   | 2 $\mu$ l        | 10 $\mu$ l       |
| 10 mM dNTPs               | 0,4 $\mu$ l      | 2 $\mu$ l        |
| 10 $\mu$ M Forward Primer | 0,4 $\mu$ l      | 2 $\mu$ l        |
| 10 $\mu$ M Reverse Primer | 0,4 $\mu$ l      | 2 $\mu$ l        |
| Template DNA              | variable         | variable         |
| Taq DNA Polymerase        | 0,66 $\mu$ l     | 3,3 $\mu$ l      |
| 25 mM MgCl <sub>2</sub>   | 1,2 $\mu$ l      | 6 $\mu$ l        |
| Nuclease Free Water       | Up to 25 $\mu$ l | Up to 50 $\mu$ l |

Table 4.6. Thermo-cycling conditions for colony PCR reaction.

| Step                 | Temperature                    | Time               |
|----------------------|--------------------------------|--------------------|
| Initial denaturation | 95 °C                          | 30 seconds         |
| (25-35 cycles)       |                                |                    |
| Denaturation         | 95 °C                          | 5 seconds          |
| Annealing            | Primer specific T <sub>m</sub> | 30 seconds         |
| Elongation           | 72 °C                          | 60 seconds per 1kb |
| Final extension      | 72 °C                          | 5 minutes          |
| Hold                 | 4 °C                           | $\infty$           |

#### 4.1.10. Analytic Digestion

After colony PCR, positive colonies were picked with sterile tips and put into the LB media containing antibiotic(s). Bacteria were grown at 37 °C for ON. Then, plasmid isolation protocol was performed. Isolated plasmid DNA was digested using

restriction enzyme(s) that provides useful information about the correct size of plasmid construct and correct orientation of insert in the vector plasmid. Digestion protocol was performed same as restriction digestion protocol which was mentioned before.

#### **4.1.11. Sequencing of plasmid constructs**

Each construct was checked using Sanger sequencing. 10  $\mu$ g sample/reaction was prepared. Service of sequencing was purchased from Macrogen Inc, Korea. Generally, universal primers for plasmid construct were chosen.

### **4.2. Cloning Strategies**

#### **4.2.1. pC3 tH5 (69-133 aa) hASC plasmid**

The original plasmid (vector plasmid) backbone was pmCherry C3 hASC plasmid which was originated from the pEGFP-C3 plasmid. The sequence of truncated H5 (69-133aa) was amplified with PCR using primers (Table 3.10). Then, PCR constructs and vector plasmid was cut by using NheI and HindIII enzymes. Then restricted constructs were ligated and then, positive colonies were picked and send them to ensure newly generated plasmid constructs including tH5 sequence.

#### **4.2.2. Site-Directed Mutation on pC3 tH5(69-133 aa) hASC plasmid**

The pC3 tH5(69-133 aa) hASC plasmid was successfully generated. Then, western blot experiment was performed to see protein expression of truncated H5-ASC. Unexpectedly, there were 2 bands which one of them was correct but the other was around 22 kDa. Then, we hypothesized that this band should belong to ASC protein itself. So, site-directed mutagenesis (SDM) was performed on the 'ATG' sequence of ASC coding sequence in the plasmid. Briefly, Q5 reaction buffer which was shown in Table 4.7 for SDM was prepared without the template, forward and reverse primers. Reaction buffer was divided into two different PCR tubes and recommended primers were added into separate tubes. Lastly, template DNA was added into each tube and

2 step PCR protocol was performed according to Table 4.8. After 10 cycles, tubes containing buffers and template were mixed in 1 PCR tube and PCR was run again for 15 cycles. After the PCR reaction was done, DpNI (NEB, USA) digestion was performed. According to PCR amount, recommended Cut Smart buffer and DpNI were added as seen in Table 4.9. Then, a small amount of DNA was loaded into agarose gel to control only 1 band was observed at the expected size and remaining DNA in PCR tube was extracted by performing PCR Clean-up protocol according to manufacturer's instructions (MN, NucleoSpin Gel and PCR Clean-up, Germany). The newly generated plasmid product was transformed into Stbl3 strain of *E. coli* and positive colonies were picked the next day.

Table 4.7. Reaction components of PCR reaction for corresponding volume for SDM.

| Component                       | 25 $\mu$ l Reaction | 50 $\mu$ l Reaction | Final [C]       |
|---------------------------------|---------------------|---------------------|-----------------|
| 5X Q5 Reaction Buffer           | 5 $\mu$ l           | 10 $\mu$ l          | 1X              |
| DMSO                            | 1,5 $\mu$ l         | 3 $\mu$ l           |                 |
| 10 mM dNTPs                     | 0,5 $\mu$ l         | 1 $\mu$ l           | 200 $\mu$ M     |
| 10 $\mu$ M Forward Primer       | 1,25 $\mu$ l        | 2,5 $\mu$ l         | 0,5 $\mu$ M     |
| 10 $\mu$ M Reverse Primer       | 1,25 $\mu$ l        | 2,5 $\mu$ l         | 0,5 $\mu$ M     |
| Template DNA                    | variable            | variable            | $\leq 1000ng$   |
| Q5 High-Fidelity DNA Polymerase | 0,25 $\mu$ l        | 0,50 $\mu$ l        | 0.02 U/ $\mu$ l |
| 5X Q5 High GC Enhancer          | 5 $\mu$ l           | 10 $\mu$ l          | 1X              |
| Nuclease Free Water             | Up to 25 $\mu$ l    | Up to 50 $\mu$ l    | -               |

Table 4.9. Protocol for a DpNI digestion experiment.

|                                 |                     |
|---------------------------------|---------------------|
| Restriction Enzyme (DpNI)       | 1 $\mu$ l           |
| 10X Cut Smart Buffer            | 6 $\mu$ l           |
| PCR Reaction Volume             | 50 $\mu$ l          |
| Incubation Time and Temperature | 3 hours at 37 °C    |
| Heat Inactivation               | 20 minutes at 80 °C |

Table 4.8. Thermo-cycling conditions for 2 steps PCR reaction.

| Step                              | Temperature                    | Time                    |
|-----------------------------------|--------------------------------|-------------------------|
| Initial denaturation              | 98 °C                          | 30 seconds              |
| (1st: 10 cycles - 2nd: 15 cycles) |                                |                         |
| Denaturation                      | 98 °C                          | 30 seconds              |
| Annealing                         | Primer specific T <sub>m</sub> | 30 seconds              |
| Elongation                        | 72 °C                          | 2.5 minutes             |
| Final extension                   | 72 °C                          | 5 minutes (1 kb/1 min.) |
| Hold                              | 4 °C                           | ∞                       |

#### 4.2.3. pet30a H1 plasmid

The original plasmid (vector plasmid) backbone was pet30a(+). The sequence of H1 was amplified with PCR using primers (Table 3.13). Then, PCR constructs and vector plasmid was cut by using EcoRV and XhoI enzymes. Restricted constructs were ligated and then, positive colonies were picked and send them to ensure newly generated plasmid constructs including H1 sequence.

#### 4.2.4. pcDNA3.1(+) H5 plasmid

The original plasmid (vector plasmid) backbone was pcDNA3.1(+). The sequence of H5 was extracted from pet30a H5 plasmid. Insert sequence of H5 and vector plasmid was cut by using HindIII enzyme. 1  $\mu$ l of CIP was added to the tube containing vector plasmid to prevent the self-ligation of vector plasmid for further ligation step. Restricted constructs were ligated and then, positive colonies were picked and send them to ensure newly generated plasmid constructs including H5 sequence.

### **4.3. Western Blot Analysis**

#### **4.3.1. Sample Preparation from Cell Culture or Isolated Protein Samples**

Cell culture samples were collected in 2X Laemmli Buffer using cell scraper. Collected cells were transferred into microcentrifuge tubes and they were denatured at 95 °C for 10 minutes and stored at -20 °C for long term storage. Isolated protein samples were transferred into microcentrifuge tubes, required amount 5X Laemmli buffer (final concentration should be at least 1X) was added and they were denatured at 95 °C for 5 minutes and stored at -20 °C for long term storage.

#### **4.3.2. Sample Preparation from Animal Tissue**

Tissues were cut into small pieces and collected in 15 ml falcons. 2 ml of 1X filtered PBS was added for each sample and samples were lysed via sonication for 30 seconds, 50% cycle, 50% power on ice. This step was repeated 3 times. Samples were incubated on ice 10 minutes then tissue lysates were centrifuged at 13000 rpm for 15 minutes to remove non-lysed and larger particles. Supernatant for each was taken into new microcentrifuge tubes and some amount of samples were aliquoted and stored at -20 °C for long term storage. Remaining was denatured with 5X Laemmli buffer at 95 °C for 10 minutes.

#### **4.3.3. Preparation of SDS-PAGE Gels**

I routinely used 15% resolving gel and 4% stacking gel for my experiments. Briefly, 8 ml of pre-prepared 15% resolving gel was poured into a falcon and was mixed with 8  $\mu$ l of TEMED and 80  $\mu$ l of 10% APS. Then, falcon was inverted 3-5 times. After well mixing, this gel solution was poured into a gel cassette. 500  $\mu$ l of 2-propanol was added on the top of the gel layer to ensure a flat surface. After polymerization of resolving gel, 2-propanol was removed and stacking gel was prepared. 4 ml of pre-prepared 4% stacking gel was poured into a falcon and was mixed with 4  $\mu$ l of TEMED and 40  $\mu$ l of 10% APS. Then, falcon was inverted 3-5 times. After well mixing, this gel solution

was poured onto the resolving gel. As fast as possible, 10 or 15 wells comb was placed. After polymerization of stacking gel, SDS-PAGE gel was ready to use.

#### **4.3.4. Protein Gel Electrophoresis**

SDS-PAGE gels were placed into the tank and inside the area between two gels were filled with fresh 1X Running Buffer, remaining area was filled with used 1X Running Buffer. Carefully, combs were taken out and 30-50  $\mu$ l of samples were loaded into the wells. Samples were run at 80 V until they passed the stacking gel. Then voltage was increased to 130 V, samples were run until the dye passed out the gels.

#### **4.3.5. Semi-dry Transfer**

Proteins which were on SDS-PAGE gels were transferred to nitrocellulose membrane. Briefly, the nitrocellulose membrane was activated by methanol for 1 minute then wash with dH<sub>2</sub>O. 2 whatmann papers and the membrane were put into transfer buffer and were getting fully wet. 1 whatmann paper, the membrane, the gel and the other whatmann paper were placed on semi-dry transfer plate respectively. Using a roller, bubbles were removed when each of them was placed separately. The cover of the semi-dry transfer machine was closed carefully and the semi-dry transfer was performed at 10 V for 1 hour.

#### **4.3.6. Membrane Blocking**

After transfer of proteins from the gel to the membrane was finished, the membrane was taken into a clean container containing 5% (w/v) non-fat dry milk in TBS-T or 5% (w/v) BSA in TBS-T to block the non-specific binding of antibodies. Membrane blocking was performed on a shaker for at least 1 hour.

#### 4.3.7. Incubation with Antibodies

Primary antibodies were prepared in 5% (w/v) BSA in TBS-T with 0.01% sodium azide to avoid microbial contamination for short term storage at 4 °C. Dilution of antibody was determined according to instructions of the manufacturer. After blocking, the membrane was washed with TBS-T for 5 minutes, 3 times. Then, the membrane was taken into a container or a falcon containing primary antibody solution. It was incubated at 4 °C for overnight on a shaker. The next day, the membrane was washed with 5% (w/v) non-fat dry milk in TBS-T for 10 minutes, 3 times and incubated with proper secondary antibody (1:4000) solution in TBS-T for at least 1 hour. Then, it was washed with TBS-T for 10 minutes, 3 times and the membrane was incubated in TBS-T until the visualization with SynGene machine.

#### 4.3.8. Visualization of Proteins

After washing of the membrane, the membrane was visualized with 400  $\mu$ l of 1:1 mixed ECL. Proteins on the membrane were visualized by using SynGene GBox machine. Briefly, the machine was turned on. Chemi-blot option was selected. Then, membrane size and ECL options with visualized marker were selected. The membrane was placed on to the black plate and the membrane was captured to visualize the proteins.

#### 4.3.9. Elution of Proteins from SDS-PAGE

After gel electrophoresis, a piece of gel including a ladder was cut and this piece was stained with commassie staining for 10 minutes. During this time, dH<sub>2</sub>O was added on top of remaining gel to prevent drying of the gel. After coomassie staining, this piece of the gel was destained with destaining buffer. After the bands were clear to observe, this piece was placed on the correct side of the remaining gel. The molecular weight of the interested protein was determined and the gel only containing the correct protein was cut and excised into small pieces and collected into a 15 ml falcon. 1 ml of elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5) for 1 gel

was added and the sample was incubated in a shaker at 30 °C overnight. Then, the sample was centrifuged at 5000g for 10 minutes. The supernatant was carefully taken into a new microcentrifuge tube.

## 4.4. Cell-Culture

### 4.4.1. ASC Speck Purification

4.4.1.1. Maintenance of HEK293FT Cell Line. HEK293FT cells were maintained in DMEM including 10% FBS, 1X MEM-NEAA, 1X Pen/Strep. When the confluence of cells was reached to 90%, the old medium in the plates were removed, the plates were washed with 3 ml/150 mm<sup>3</sup> plate 1X PBS, cells were trypsinized with 3 ml/150 mm<sup>3</sup> plate trypsin at 37 °C for 2 minutes. Trypsin was neutralized with 1:1 amount of supplemented DMEM. Cells were centrifuged at 2000 rpm for 2 minutes, media was thrown away and cells were suspended in DMEM and sub-cultured to plates.

4.4.1.2. Plasmid Isolation. Isolation of plasmid DNA was performed according to instructions of the manufacturer (MN, NucleoBond Xtra Midi EF, Germany). Briefly, 400-450 ml liquid culture of bacteria (including kanamycin) was incubated on a shaker at 37°C for ON. Then, the bacteria culture was centrifuged at 6500g for 10 min. The supernatant was thrown away and the recommended amount of buffers were added and protocol for each step was performed according to instructions of the manufacturer respectively. Lastly, plasmid DNA was suspended with 1500 µl of dH<sub>2</sub>O-EF. The yield of plasmid DNA and the ratios of 260/280 and 260/230 for plasmid DNA was calculated by using a spectrophotometer.

4.4.1.3. Calcium Phosphate Transfection. HEK293FT cells were transfected via calcium phosphate method. Ingredients were shown in Table 4.10. Briefly, ddH<sub>2</sub>O and plasmid DNA were mixed and ice cold 2X CaCl<sub>2</sub> was added drop by drop. After 5 minutes incubation at RT, 2X HBS buffer was added and was mixed immediately until the bubbles were formed. After 5 minutes incubation at RT, the mixture was added

onto plates drop by drop. Cells were harvested after 48 hours transfection.

Table 4.10. Ingredients for calcium-phosphate transfection method for 1 sample.

| Plate        | Seed Cells                                 | DNA           | ddH <sub>2</sub> O | 2X CaCl <sub>2</sub> | 2X HBS       |
|--------------|--------------------------------------------|---------------|--------------------|----------------------|--------------|
| 6 well plate | 8x10 <sup>5</sup> -1x10 <sup>6</sup> /well | 0.1-1 $\mu$ g | 219.5 $\mu$ l      | 30.5 $\mu$ l         | 250 $\mu$ l  |
| 100 mm plate | 4-6x10 <sup>6</sup> /plate                 | 5-10 $\mu$ g  | 439 $\mu$ l        | 61 $\mu$ l           | 500 $\mu$ l  |
| 150 mm plate | 16-18x10 <sup>6</sup> /plate               | 30-40 $\mu$ g | 1317 $\mu$ l       | 183 $\mu$ l          | 1500 $\mu$ l |

4.4.1.4. Production and Isolation of ASC Specks. HEK293FT cells were transfected with 30  $\mu$ g of p-C3-mCherry-ASC and 40  $\mu$ g of p-C3-tOVA(1-238aa)-ASC plasmids separately. Also, 18  $\mu$ g of p-C3-mCherry-ASC and 36  $\mu$ g p-C3-tH5(69-133aa)-ASC plasmids were co-transfected in HEK293FT cells. After 48 hours transfection, old media from the plate were thrown away. Plates were washed with 3 ml 1X PBS. After washing, 3 ml 1X PBS was added to each plate and cells were collected by cell scraper and put 3 plates of cells into 1 of 50 ml falcon. Then cells were sonicated for 3 times [5 seconds, 7 cycles, 50%power] on ice. Samples were centrifuged at 2400 g for 5 minutes at RT. After that, samples were vortexed for 5 minutes and incubated at RT for 5 minutes for gravity sedimentation. Supernatant for each was collected in a new falcon and centrifuged at 400 g for 5 minutes (p-C3-mCherry-ASC and p-C3-tOVA(1-238aa)-ASC transfection) or centrifuged at 500 g for 5 minutes (p-C3-mCherry-ASC and p-C3-tH5(69-133aa)-ASC co-transfection). The supernatant was removed and 10 ml 1X PBS was added for each falcon. From vortexing step to last mentioned centrifugation step, the protocol was repeated again for 6-8 times. This depends on the number of plates, briefly, repetition was performed until no filaments were observed. Then, ASC specks were suspended in 1X PBS and kept at 4 °C. The purity of ASC specks was determined with coomassie blue staining and the amount of ASC specks were determined via BCA method. All steps except sonication were performed at cell culture in sterile conditions.

4.4.1.5. Determination of Amount of ASC Specks. Amount of ASC specks were determined via BCA assay. Briefly, standards were prepared according to instructions of the manufacturer (Pierce<sup>TM</sup> BCA Protein Assay Kit, Thermo). Standards were kept

at -20 °C for long term storage. 5  $\mu$ l of each standard and samples were added into a 96 well plate separately. 25  $\mu$ l of working reagent (for 1 ml of reagent A, 20  $\mu$ l of reagent S was added) was added. Then, 200  $\mu$ l of reagent B was added in dark and 96 well plate was covered with an aluminum coil. The plate was incubated at RT for 15 minutes. Then the absorbance was measured at 750 nm on the plate reader.

#### **4.4.2. Effect of ASC Specks on THP-1 Cells**

4.4.2.1. Maintenance of THP-1 Cell Line. THP-1 cell line was kindly provided by Prof. Ahmet Gul from Istanbul University. eGFP-ASC stable THP-1 cell line was constructed via lentiviral transduction of monocytes with pLenti-Efla-EGFP-ASC by Ali Can Sahillioglu (M.Sc.). THP-1 and eGFP-ASC stable THP-1 cells were maintained in RPMI 1640 including 10% FBS, 1X MEM-NEAA, 1X Pen/Strep. When the confluence of cells was reached to 90%, cells were collected in a falcon and centrifuged at 300g for 2 minutes, media was thrown away and cells were suspended in supplemented RPMI 1640 and sub-cultured to flasks.

4.4.2.2. Differentiation of the THP-1 Monocytes to Macrophages.  $1.5 \times 10^6$ /well cells in a 6 well plate were differentiated with 100 nM/ml PMA containing supplemented RPMI 1640. After 12 hours, the media for each well was thrown away and refreshed with fresh supplemented RPMI 1640. Cells were incubated at 37 °C for 36 hours.

4.4.2.3. Treatment of the THP-1 Macrophages with ASC Specks. THP-1 cells were treated with mCherry ASC and tH5 ASC specks respectively. The medium of cells was collected at different time points (12, 18 and 24 hours later after the treatment). The number of macrophages that engulfed ASC specks was determined via Flow Cytometry. Macrophages were collected via trypsin in a falcon. Cells were centrifuged at 300 g for 5 minutes then suspended in 1X PBS. The number of events was chosen as 10000 and analysis was performed.

4.4.2.4. Cytokine ELISA after the Engulfment of ASC Specks. Using mediums which were collected at different time points, sandwich ELISA protocol was performed according to instructions of the manufacturer (RD, Duo Set Human ELISA, Germany). Briefly, 96 well ELISA plate was covered with 100  $\mu$ l of capture antibody diluted in PBS. The plate was sealed and incubated at RT for ON. Each well was aspirated and washed with 300  $\mu$ l of wash buffer (PBS-T) for 3 times. After the last wash, remaining wash buffers and bubbles were removed by blotting the plate against clean towel papers. 300  $\mu$ l of reagent diluent (1% BSA in PBS) was added to each well and the plate was incubated at RT for a minimum of 1 hour. The washing step was repeated. After the last wash, remaining wash buffers and bubbles were removed by blotting the plate against clean towel papers. 100  $\mu$ l of standards or samples were added and the plate was covered with stretch film and incubate at RT for 2 hours. The washing step was repeated and after the last wash, the same cleaning protocol was performed. 100  $\mu$ l of detection antibody was added to each well and the plate was covered with stretch film and incubate at RT for 2 hours. The washing step was repeated and after the last wash, the same cleaning protocol was performed. 100  $\mu$ l of Streptavidin-HRP was added to each well avoiding the plate from the direct light and the plate was covered with stretch film and incubate at RT for 20 minutes. The washing step was repeated and after the last wash, the same cleaning protocol was performed. 100  $\mu$ l of substrate mixture (1:1 mixture of color reagent A and B) was added to each well avoiding the plate from the direct light and the plate was incubated at RT for 20 minutes. Then, 50  $\mu$ l of stop solution (2N H<sub>2</sub>SO<sub>4</sub>) was added to each well. The plate was gently tapped to ensure thorough mixing. The absorbance of each well was measured at 450 and 540 nm via plate reader.

## **4.5. Immunization of Mice with ASC Specks against H5N1 Influenza**

### **4.5.1. Animals**

8-10 weeks old C57BL/6 mice were used for experiments. Experiments were performed in Vivarium at Bogazici University. All experimental procedure was approved by the Committee of Animal Ethics of Bogazici University.

#### **4.5.2. Immunizations**

Animals were divided into 4 different immunization groups. Each group included 5 mice. Mice were immunized with only 1X PBS, only mCherry-ASC specks, Alum with tH5 and lastly mCherry ASC with tH5 ASC specks. 300  $\mu$ l of 1X filtered PBS, 100  $\mu$ g of mCherry-ASC specks, 15  $\mu$ g of tH5 in 1X Alum diluted in PBS and 50  $\mu$ g of tH5 ASC (including 15  $\mu$ g tH5) was intraperitoneally injected for each group of mice.

#### **4.5.3. Blood Collection**

7 days after the first immunization, first collection blood for each mice was done. 14 days after the first immunization, the second immunization was performed. 3 days after the second immunization, the blood of each mouse collected. 14 days after the second immunization, the third immunization was performed. 3 days after the third immunization, the blood of each mouse collected. Briefly, mice were anesthetized with isoflurane and 100-150  $\mu$ l of blood was collected in 1.5 ml micro-centrifuge tube by bleeding from the orbital sinus of the animals via heparinized capillary tubes. Collected blood samples were incubated at 37 °C for 1 hour then, they were centrifuged at 11000 rpm for 1 minute. Serum of blood was collected into new tubes and kept at -80 °C for long term storage.

#### **4.5.4. Tissue Collection**

3 days after the last immunization, mice were sacrificed and the blood of each mice was collected from the hearts of them. Besides, the spleen of them was collected and kept at -80 °C for long term storage.

#### **4.5.5. ELISA for H5 Specific IgG Response**

H5 specific IgG response was determined by ELISA. Briefly, 96 well ELISA plate was covered with 200 ng/well of H5 protein diluted in 100  $\mu$ l of PBS and 3 of wells were used for negative control which was included only PBS. The plate was sealed

and incubated at 4 °C for ON. Each well was aspirated and washed with 300  $\mu$ l of wash buffer (PBS-T) for 3 times. After the last wash, remaining wash buffers and bubbles were removed by blotting the plate against clean towel papers. 200  $\mu$ l of reagent diluent (1% BSA in PBS) was added to each well and the plate was incubated at RT for minimum 1 hour, maximum 2 hours. The washing step was repeated. After the last wash, remaining wash buffers and bubbles were removed by blotting the plate against clean towel papers. 100  $\mu$ l of standards (from 100 pg/ml to 8000 pg/ml), or samples (1:500 dilution) were added and the plate was covered with stretch film and incubate at RT for 1 hour. The washing step was repeated and after the last wash, the same cleaning protocol was performed. 100  $\mu$ l of 1:2000 anti-mouse IgG HRP linked secondary antibody was added to each well and the plate was covered with stretch film and incubate at RT for 1 hour. The washing step was repeated and after the last wash, the same cleaning protocol was performed. 100  $\mu$ l of substrate mixture (1:1 mixture of color reagent A and B) was added to each well avoiding the plate from the direct light and the plate was incubated at RT for 20 minutes. Then, 20  $\mu$ l of stop solution (2N H<sub>2</sub>SO<sub>4</sub>) was added to each well. The plate was gently tapped to ensure thorough mixing. The absorbance of each well was measured at 450 and 540 nm via plate reader.

#### **4.6. The Hemagglutination Assay for H5 Antigen**

##### **4.6.1. Preparation of 0 type Human Blood**

1.5 mg EDTA per ml of blood was used as anti-coagulant. 5 ml blood was collected in a 50 ml falcon including EDTA and mixed well. Then the falcon was centrifuged at 2000 rpm for 10 minutes. Serum was discarded and the pellet was resuspended with 5 ml of 1X filtered PBS and centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded. This washing step was repeated 2 more times. After last wash, 1 ml of the pellet was diluted in 9 ml 1X filtered PBS to prepare 10% stock solution. Then, using this stock solution, 0.5% working solution was prepared.

#### 4.6.2. Preparing Cells and Hemadsorption Protocol

24 hours before doing the experiment,  $1 \times 10^6$  HEK293FT and HeLa cells were transfected with 100 ng pcDNA3-H5 plasmid in a 6 well plate. After 24 hours of transfection, the medium in the wells was thrown away. The transfected cells were washed with 1 ml of 1X PBS for 3 times. Then, cells were fixed with 4% PFA for 15 minutes. After fixation, cells were washed with 1 ml of 1X PBS for 3 times. 0.5% working solution of RBC was added and the plate was incubated at 4 °C for 60 minutes. Then, carefully wash the cells with 1 ml of 1X PBS for 3 times. Between each wash, the plate was incubated at RT for 2 minutes. Lastly, PBS in each well was discarded and wells were looked under the bright field microscope.

### 4.7. EG7-OVA Tumor Model

#### 4.7.1. Maintenance of EG7-OVA Cell Line

EG7-OVA cell line was kindly provided by Prof. Ihsan Gursel from Bilkent University. EG7-OVA cell line is a derivative of EL-4 which is C57BL/6 mouse lymphoma cell line. This cell line was stably transfected with pAc-neo-OVA plasmid and secrete OVA constitutively. EG7-OVA cells were maintained in RPMI 1640 including 10% FBS, 1X MEM-NEAA, 1X Pen/Strep, 0.05 mM 2-mercaptoethanol and 0.4 mg/ml G418. When the confluence of cells was reached to 90%, cells were collected in a falcon and centrifuged at 2000 rpm for 2 minutes, media was thrown away and cells were suspended in RPMI and sub-cultured to flasks.

#### 4.7.2. Inoculation of EG7-OVA and Immunizations

$2.5 \times 10^6$  EG7-OVA cells in 100  $\mu$ l 1X PBS were inoculated for each mice. Before inoculation, OVA expression of these cells was checked with western blot analysis using anti-OVA antibody. Cells were prepared under sterile conditions. Inoculation of these cells was performed to the right dorsal flank of each mice. When tumors reached a palpable size ( $\geq 50 \text{ mm}^3$ ) after 12-14 days, intraperitoneal injections were performed

as follows; only 1X PBS, 100  $\mu$ g of OVA, 100  $\mu$ g of OVA with 100  $\mu$ g of mCherry ASC and 100  $\mu$ g of tOVA ASC in 300  $\mu$ l of 1X PBS. Size of tumors was determined with a caliper. The volume of each tumor was calculated according to a formula;  $\frac{1}{2}[(\text{short length}^2) \cdot \text{long length}]$ . Second injections were repeated as same as the first injections. The experiment was finished 1 week after second injections because most of the tumors of mice which immunized with tOVA ASC were eradicated. Tumors of animals were calculated via caliper. Then, all animals were sacrificed. Blood of them was collected from the hearts of them. Also, spleens and livers of them were collected for further analysis. Tumors of animals were excised and embedded in OCT. All tissues were kept on ice until the end of the experiment for the last mouse. Collected blood samples were incubated at 37 °C for 1 hour then, they were centrifuged at 11000 rpm for 1 minute. Serum of blood was collected into new tubes and kept at -80 °C for long term storage.

#### **4.7.3. Blood Collection**

3 days after the last immunization, the blood of each mouse collected. Briefly, mice were sacrificed and 1000  $\mu$ l of blood was collected in 1.5 ml micro-centrifuge tube by direct syringing from hearts of the animals via syringe. Collected blood samples were incubated at 37 °C for 1 hour then, they were centrifuged at 11000 rpm for 1 minute. Serum of blood was collected into new tubes and kept at -80 °C for long term storage.

#### **4.7.4. Tissue Sampling from Immunized Mice**

After 3 days from the last immunization, animals were sacrificed. Spleens, livers and lymph nodes were excised and collected. Livers and lymph nodes were kept at -80 °C for long term storage to use them for further western blot experiments. Spleens were used for isolation of splenocytes to use them for further ex-vivo recall assay.

#### 4.7.5. Isolation of splenocytes

Spleens of mice were smashed using plunger of a sterile syringe in 3 ml RPMI 1640 medium supplemented with 20%FBS. The media from each spleen including cells not remaining tissue was collected into 15 ml sterile falcons and centrifuged at 300g for 10 minutes at RT. Supernatants were discarded. Red blood cells were lysed using 2 ml of ACK lysis buffer for each falcon at RT for 5 minutes. Cells were washed with 1X sterile PBS, then centrifuge at 300g for 5 minutes. This washing step was repeated 1 more time. Then cells were suspended in 1X sterile PBS. Cells were counted with countess or flow cytometer and  $1 \times 10^6$  cells/well in 200  $\mu$ l RPMI 1640 medium supplemented with 20% FBS, 1X Pen/Strep, 1% HEPES and 1%MEM-NEAA were seeded to a 96 well plate.

#### 4.7.6. ELISA for OVA Specific IgG Response

OVA-specific IgG response was determined by ELISA. Briefly, 96 well ELISA plate was covered with 100 ng/well of OVA protein diluted in 100  $\mu$ l of PBS and 3 of wells were used for negative control which was included only PBS. The plate was sealed and incubated at 4 °C for ON. Each well was aspirated and washed with 300  $\mu$ l of wash buffer (PBS-T) for 3 times. After the last wash, remaining wash buffers and bubbles were removed by blotting the plate against clean towel papers. 200  $\mu$ l of reagent diluent (1% BSA in PBS) was added to each well and the plate was incubated at RT for minimum 1 hour, maximum 2 hours. The washing step was repeated. After the last wash, remaining wash buffers and bubbles were removed by blotting the plate against clean towel papers. 100  $\mu$ l of standards (from 25 pg/ml to 5000 pg/ml), or samples (1:500 dilution) were added and the plate was covered with stretch film and incubate at RT for 1 hour. The washing step was repeated and after the last wash, the same cleaning protocol was performed. 100  $\mu$ l of 1:2000 anti-mouse IgG HRP linked secondary antibody was added to each well and the plate was covered with stretch film and incubate at RT for 1 hour. The washing step was repeated and after the last wash, the same cleaning protocol was performed. 100  $\mu$ l of substrate mixture (1:1 mixture of color reagent A and B) was added to each well avoiding the plate from the direct

light and the plate was incubated at RT for 20 minutes. Then, 20  $\mu$ l of stop solution (2N H<sub>2</sub>SO<sub>4</sub>) was added to each well. The plate was gently tapped to ensure thorough mixing. The absorbance of each well was measured at 450 and 540 nm via plate reader.



## 5. EXPERIMENTS AND RESULTS

### 5.1. Loading of H5 antigen onto ASC specks

We have shown that cytosolic proteins that are including hydrophobic peptides could co-aggregate on the ASC speck [11]. In order to use this strategy for the development of novel vaccines, we carried out several experiments to develop a prototype vaccine against H5N1 influenza. In our work we used fusion protein constructs to minimize variability during the production of H5 loaded ASC specks.

#### 5.1.1. Cloning of tH5 to construct pC3tH5(69-133aa)-hASC plasmid

Previous lab member Alican Sahillioglu (M.Sc.) constructed pC3eGFP-hASC and pC3mCherry-hASC vector using peGFP-C3 plasmid backbone. For the development of novel vaccine against H5N1 influenza, pC3tH5(69-133aa)-hASC plasmid was constructed. The sequence of tH5(69-133aa) which is the most antigenic part of H5N1 [30] was amplified with specific primers from pet30aH5 plasmid including full length H5 coding sequence. Both insert sequence(tH5) and vector plasmid (pC3mCherry-hASC) were digested with NheI and HindIII enzymes. Restricted products were ligated by T4 ligase and the ligation products were transformed into Stbl3 competent *E. coli* bacteria strain. The transformed were plated on to Kan+ LB agar plates. Positive colonies were selected by analytic digestion with EcoRI enzyme as seen in Figure 5.1. In Fig 5.1 A, expected band size for amplified tH5 around 200 bp was observed. In Fig 5.1 B, expected band sizes for the digested products were observed. In Fig 5.1 C, 15 out of 25 colonies gave the correct size after digestion with EcoRI enzyme. Sequence of these plasmids were confirmed.

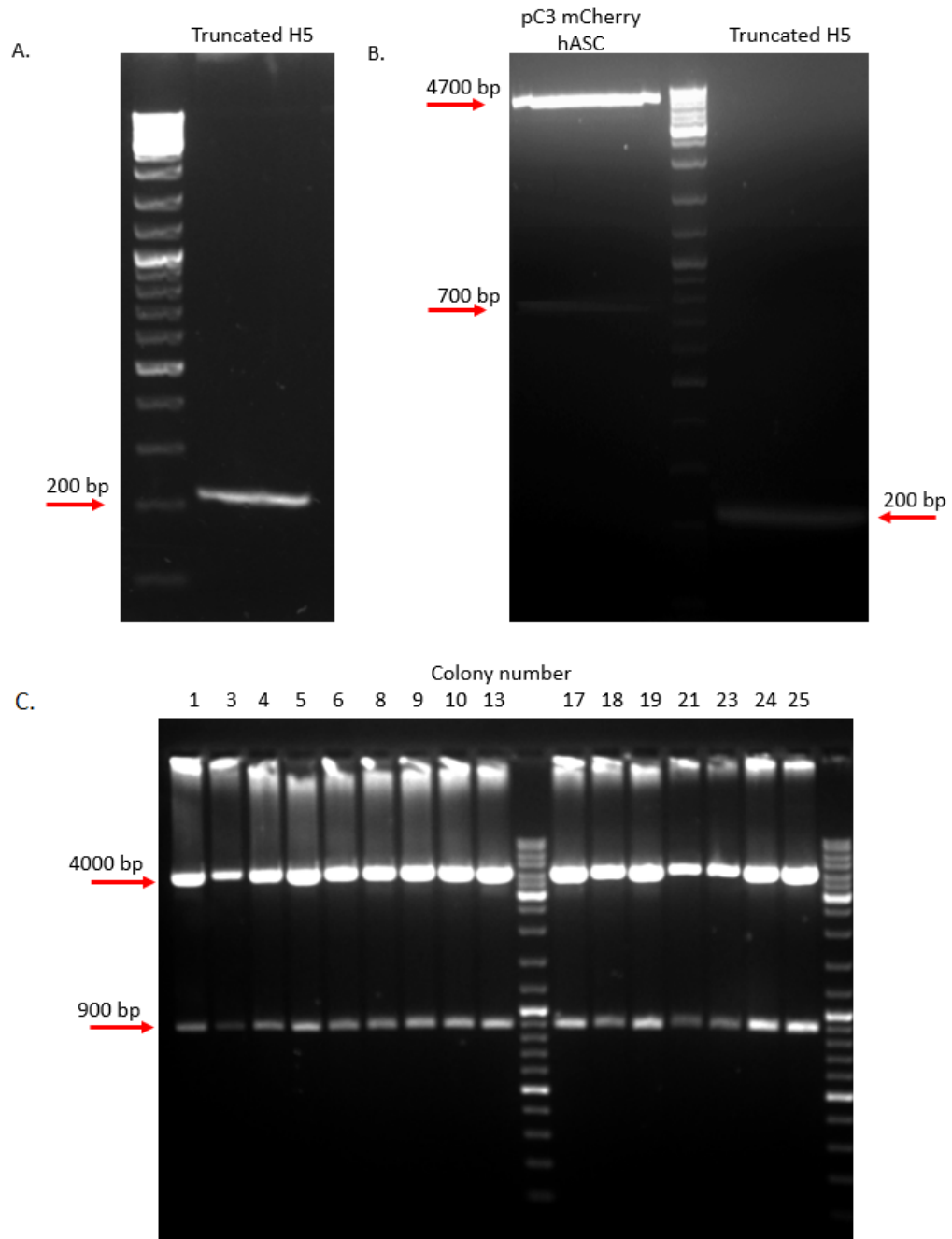


Figure 5.1. Cloning of tH5 into pC3EGFP vector backbone. A. Amplification of tH5 by PCR. B. Restriction products of insert sequence(tH5) and vector plasmid (pC3mCherry-hASC). C. Analytic digestion for selected colonies.

### 5.1.2. Site Directed Mutagenesis(SDM) on pC3tH5(69-133aa)-hASC plasmid

After pC3tH5(69-133aa)-hASC plasmid was successfully constructed, the protein expression of this plasmid was checked by Western blot experiment using monoclonal anti-hASC antibody. The western blot result showed that there were two ASC protein variants expressed including one of 29 kDa which indicates tH5-ASC and an another variant at 22 kDa which indicates only ASC protein. So, the 'ATG' sequence of hASC was changed to 'GCG' by site directed mutagenesis as seen in Figure 5.2. 8 out of 15 colonies gave the correct size after digestion with EcorI enzyme. Sequence of these plasmids were confirmed.

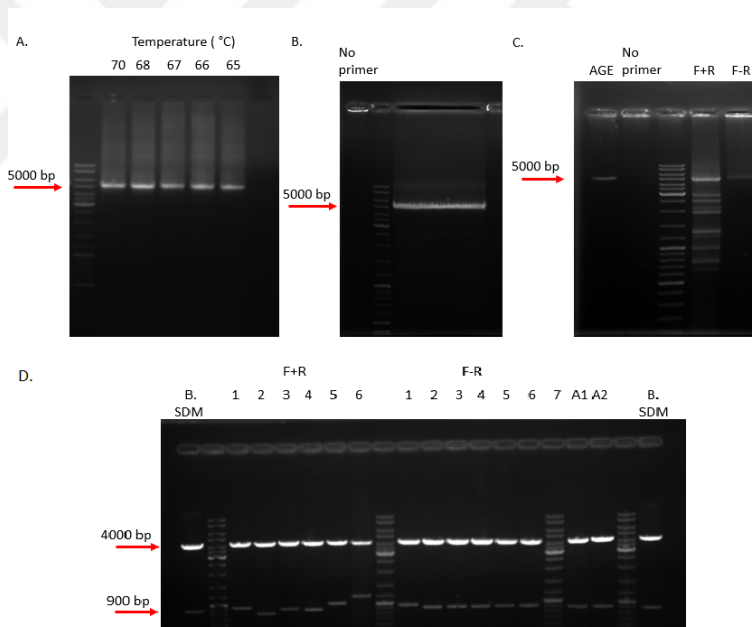


Figure 5.2. Site directed mutagenesis on hASC to construct final version of pC3tH5(69-133aa)-hASC plasmid. A. Control of SDM primers at different temperature gradients. B. Large scale amplification of SDM construct. C. Control of SDM reactions with DpNI digestion. D. Analytic digestion with EcoRI. B.SDM:

Before SDM

### 5.1.3. Expression of tH5-ASC protein after SDM on pC3tH5(69-133aa) plasmid

Newly generated pC3tH5(69-133aa)-hASC plasmid was successfully constructed after SDM, the protein expression of this plasmid was checked by Western blot experiment using monoclonal anti-hASC antibody. Western result showed that there was only one ASC protein expression including 29 kDa which indicates tH5-ASC although there were two ASC protein expression for pC3tH5(69-133aa)-hASC before SDM as seen in Figure 5.3. After SDM, colony names -4(belong to F-R group) and AGE1(belong to agarose gel extracted group) gave the correct bands at 29 kDa. Non-transfected cell lysate was used for negative control, mCherry-ASC and eGFP-ASC proteins were used as positive control. ASC proteins were observed via monoclonal anti-hASC antibody. Levels of actin were checked via anti-beta actin antibody.

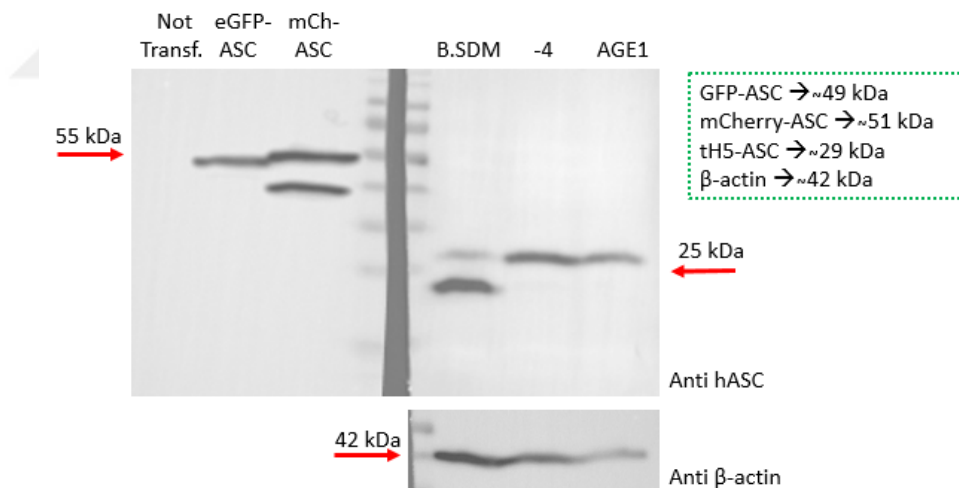


Figure 5.3. Western blot analysis of tH5-ASC expression in human cells. Before SDM experiment, there were two bands indicating 29 kDa(tH5-ASC protein) and 22 kDa(ASC protein). Not Transf.:Not transfected, mCh:mCherry, B.SDM:Before SDM

## 5.2. Purification of tH5-ASC, tOVA-ASC and mCherry-ASC specks for further studies.

For immunizations of mice; tH5-ASC specks were used as a vaccine against H5N1 influenza, tOVA-ASC specks were used as a vaccine against EG7-OVA thymoma and mCherry-ASC specks were used as a control group both of experimental groups. HEK293FT cells were co-transfected with plasmids expressing tH5-ASC and mCherry-ASC specks to get higher yields for tH5-ASC specks. Besides, HEK293FT cells were transfected with plasmids expressing mCh-ASC and tOVA-ASC separately to produce mCh-ASC and tOVA-ASC specks. These ASC specks were successfully purified and verified via coomassie blue staining as seen in Figure 5.4.

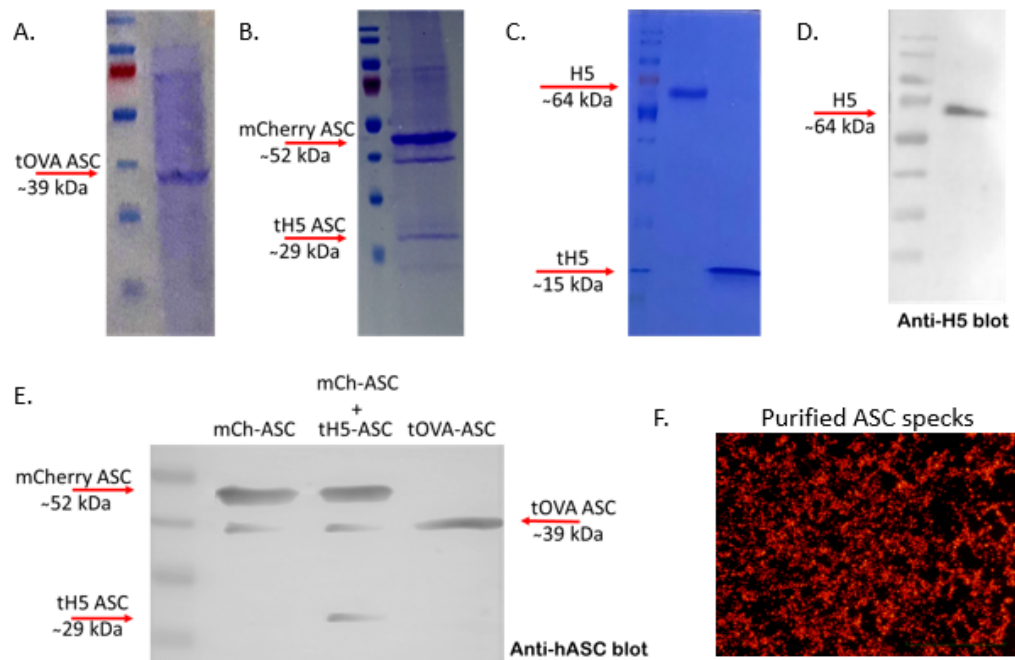


Figure 5.4. Assessment of purity for ASC specks. A. Coomassie blue staining of purified tOVA-ASC B. Coomassie staining of purified mCh-ASC and tH5-ASC. C. SDS gel purified H5 and His-Trap purified tH5. D. Gel purified H5 by anti-H5 western blotting. E. Purified ASC specks by anti-hASC blotting. F. Purified mCherry-ASC under fluorescent microscope (4X magnification)

5.3. THP-1 macrophages efficiently engulf mCherry-ASC specks.

To test the capacity of antigen delivery by ASC specks to macrophages, THP-1 macrophages were treated with 50  $\mu\text{g}/\text{ml}$  mCherry ASC specks to observe the effect of the ASC specks on antigen uptake. As seen in Figure 5.5, mCherry ASC specks were engulfed from THP-1 macrophages after being treated with these specks for 24 hours. To confirm the number of THP-1 cells which engulfed ASC specks, quantification analysis was performed via flow cytometry. Data from flow cytometry as seen in Figure 5.5B showed that approximately 52% of total cells engulfed the mCherry proteins on ASC specks. These experiments showed efficient uptake of mCherry protein on ASC specks by macrophages.

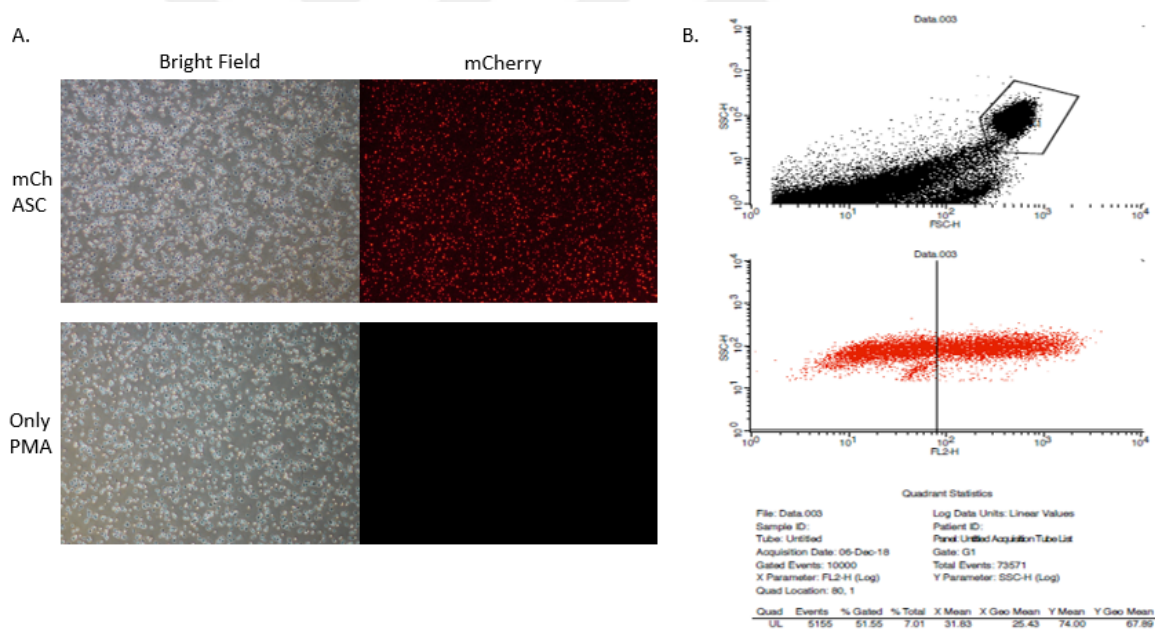


Figure 5.5. mCherry ASC specks were engulfed by PMA differentiated THP-1 macrophages. A. Flourescent images of only PMA differentiated macrophages and mCherry ASC treated macrophages after 24 hours (4X magnification). B. Flow cytometry data. mCh ASC: mCherry ASC

### 5.3.1. Proinflammatory cytokine secretion by THP-1 macrophages after phagocytosis of mCherry-ASC specks.

Pro-inflammatory cytokine response upon treatment with ASC specks was an issue to understand. To understand whether these specks cause the pro-inflammatory cytokine response after the engulfment, PMA differentiated THP-1 macrophages were treated with 50  $\mu\text{g}/\text{ml}$  mCherry ASC specks for 12 and 24 hours and supernatants of the samples were collected to check the secretions of cytokines. Secretions of IL-1 $\beta$  and TNF- $\alpha$  in the supernatants were determined by performing ELISA using Duo Set Human ELISA kit. As seen in Figure 5.6, after the engulfment of ASC specks, the secretion levels of cytokines IL-1 $\beta$  and TNF- $\alpha$  were significantly increased. The average of IL-1 $\beta$  secretions for mCh-ASC group were 641 ng/ml at 12 hours 708 ng/ml at 24 hours. The average of TNF- $\alpha$  secretions for mCh-ASC group were 2987 ng/ml at 12 hours and 1450 ng/ml at 24 hours. These results showed that engulfment of proteins on ASC specks from THP-1 macrophages can be enhanced and can lead to increase in the inflammatory response even though these proteins have not antigenic.

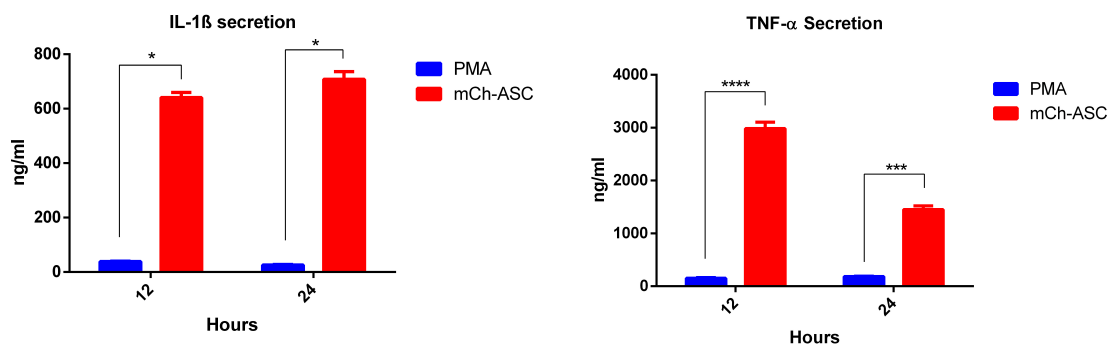


Figure 5.6. ASC-speck phagocytosis leads to secretions of IL-1 $\beta$  and TNF- $\alpha$ . mCh-ASC: Macrophages treated with mCherry ASC specks, PMA: Macrophages only differentiated with PMA, no treatment.

#### 5.4. Cloning of H5 Antigen Into an Eukaryotic Vector

Cells after being infected by certain viruses acquire the ability to bind to RBCs (hemadsorption) on their surface by the reason of interactions between ligands on RBCs and viral coat proteins which are surface-expressed on the virus-infected cells. Also, free particles of some viruses have the ability to bind to RBCs and after mixing them with RBCs, cells aggregate into a lattice of the cross linked cells. This property is known as hemagglutination and it can be useful for the quantification of viruses that have this activity for example influenza A viruses. The free virus particles of some viruses are also able to bind to red blood cells, and when mixed together will cause the cells to aggregate into a lattice of cross-linked cells. This property is called hemagglutination, and can be used as the basis for quantifying viruses that possess this activity (eg. influenza A virus). To show hemagglutination property of H5 antigen from H5N1 influenza, an eukaryotic plasmid that is expressing H5 protein was successfully constructed as seen in Figure 5.7. Red arrows shows positive colonies that gave the correct size after digestion with EcorI enzyme. Sequence of these plasmids were confirmed.

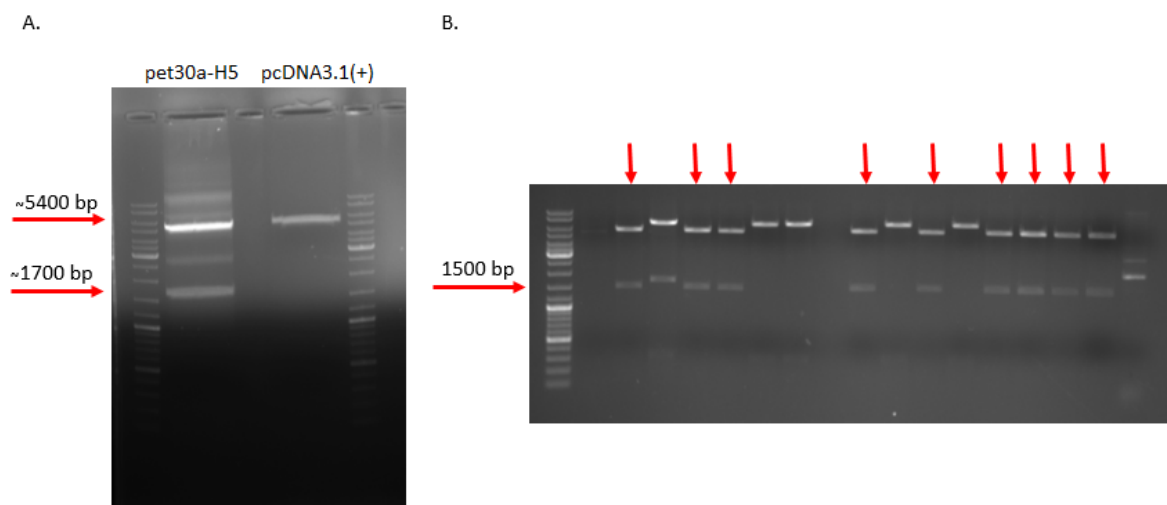


Figure 5.7. Cloning of H5 into pcDNA3.1(+) empty vector backbone. A. Restriction products of insert sequence(H5) and vector plasmid (pcDNA3.1(+)). B. Analytic digestion for selected colonies.

#### 5.4.1. Hemagglutination assay with H5 antigen expressing HEK293FT cells

Hemagglutination assay was performed to demonstrate viral antigen H5 was surface-expressed for these cells. [31] Briefly, HEK293FT cells were transfected with 100 ng pcDNA3-H5 plasmid. After 24 hours transfection, hemadsorption protocol was performed. Also, another set of transfected cells were collected with 300  $\mu$ l of 2X Laemmli buffer by using a scraper. Then, western blot analysis was performed. As seen in Figure 5.8, protein expression of H5 was demonstrated via western blot analysis using anti-H5 monoclonal antibody and hemagglutination property of H5 antigen was shown for the transfected cells compared with non-transfected cells. Non-transfected cells had no antigen on their surface so, they were not interacted to RBCs. Transfected cells had surface expressed H5 proteins so they bound to RBCs via interaction between ligands on RBCs and H5 protein on the cell surface. Red arrows show some RBCs, black arrows show some HEK293FT cells. Thus we were able to show that viral antigen H5 was surface-expressed and had hemadsorption capability.

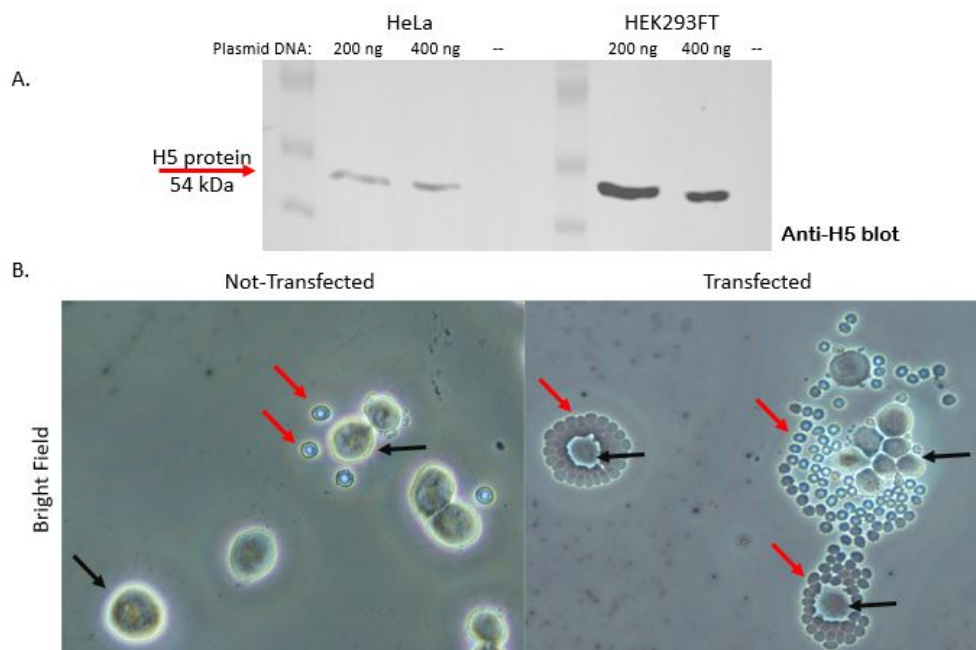


Figure 5.8. Expression of H5 after transfection with pcDNA3-H5 plasmid. A. Expression of H5 protein was shown by performing western blot. B. Hemagglutination property of H5 was demonstrated via performing hemadsorption protocol.

### 5.5. Immunization of Mice by tH5-ASC Specks against H5N1

The major aim of vaccination is to provide immunity in host organisms against antigens or microbes. This immunity can be based on cellular and/or humoral response. The humoral immune response is identified as the production of antibodies from B lymphocytes. IgG is one of the major class in immunoglobulins family and it is found in blood to provide protection against microbes or antigens. To understand whether tH5-ASC specks cause increasing in IgG response against H5N1, 6-10 weeks C57BL/6 wild type mice were used for vaccination experiment. As seen in Table 5.1, 60  $\mu\text{g}$  of tH5-ASC specks were intraperitoneally injected to 5 mice. Total of 3 injections were performed to boost the immune system strong. Figure 5.9 shows the timeline for each boosted injections.

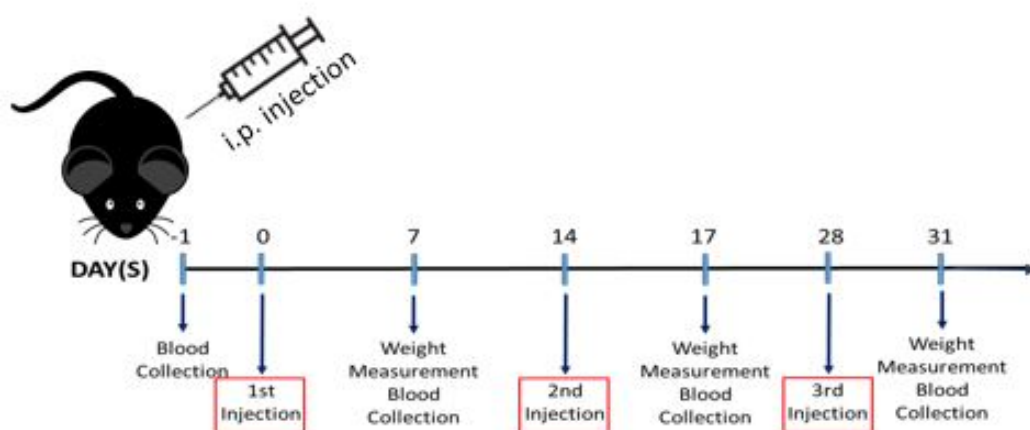


Figure 5.9. Vaccination timeline for immunization of mice.

In addition to immunization with tH5-ASC specks, 1X PBS injected 5 mice were used as a negative control, 100  $\mu\text{g}$  of mCherry ASC specks injected 5 mice were used as the control for antigen specificity and Alum with tH5 injected 5 mice were used as control adjuvant ability of ASC specks comparing with known antigen Alum. Importantly, the blood of each mouse was collected before any injection to determine basal IgG response. For each blood collection, plasma samples of mice blood were collected and they were used to perform sandwich ELISA.

Table 5.1. Injection Groups

| Groups   | Amount of tH5 protein |
|----------|-----------------------|
| 1X PBS   | 0                     |
| mCh-ASC  | 0                     |
| tH5-ASC  | 15                    |
| Alum+tH5 | 15                    |

### 5.5.1. H5 Specific IgG Response Against tH5-ASC Specks

To see the effect of tH5-ASC specks as a vaccine and adjuvant, the total IgG response from each mice was determined via sandwich ELISA. There were four experimental groups; PBS, mCh-ASC, Alum+tH5 and tH5-ASC+mCh-ASC. PBS was used as a negative control because ASC specks were given in PBS. tH5-ASC specks were obtained with mCh-ASC specks after co-transfection of HEK293FT cells with pC3tH5-ASC and pC3mCh-ASC plasmids to increase the yield of tH5-ASC production and purification. mCh-ASC group was another group because tH5-ASC specks were given to mice with mCh-ASC and also this group used as control group for antigen specificity.

As seen in Figure 5.10, Before immunizations, mice had a basal level of IgG response. 7 days after first immunization, although PBS and mCh-ASC group's IgG response were not changed, H5 specific antigen response for both tH5-ASC+mCh-ASC and Alum+tH5 groups were increased slightly. 3 days after the second immunization, almost the same pattern was observed like IgG response after the first immunization. As clearly seen in the result, 3 days after last(third) immunization, tH5-ASC+mCh-ASC group had the highest score for the H5 specific IgG response although other groups had the same pattern as 3 days after second immunization result. So, we confirmed that tH5-ASC specks had an increase in the level for the response of H5 specific IgG response comparing to other groups. Although Alum was used as a model adjuvant in our experiment, mice immunized with tH5-ASC specks showed higher IgG levels compared to mice immunized with Alum+tH5.

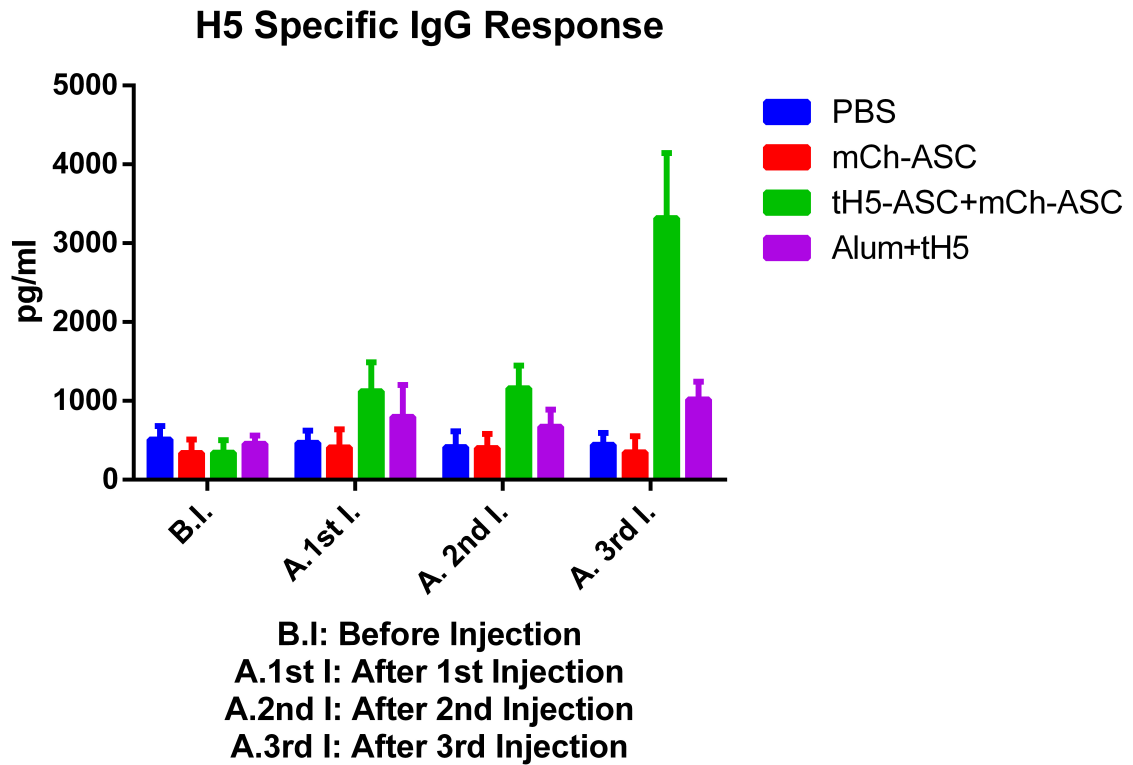


Figure 5.10. H5 antigen specific IgG response for experimental groups. Before any injection, groups had similar basal level for H5 specific IgG response. After immunizations, mice immunized with tH5-ASC+mCh-ASC had the highest level for the IgG response compared to other groups. (n=5 for each group)

### 5.6. Cloning of H1 Antigen Into Pet30a(+) Vector

In addition to the development of a vaccine against H5N1 influenza, we also want to develop another vaccine against H1N1. To develop a vaccine using ASC specks, firstly, we tried to construct H1 expressing bacterial plasmid vector. We had cDNA of H1 antigen protein. Specific primers were designed and the H1 sequence was amplified by PCR. Then, amplified PCR product of H1 and vector plasmid pet30a(+) were restricted by *EcoRV* and *XhoI*. After that, the ligation protocol was performed. Ligation products were transformed into *Stbl3* competent *E. coli* bacteria strain. Positive colonies were selected by colony PCR as seen in Figure 5.11. After colony PCR, the

sequence of the plasmids was confirmed.

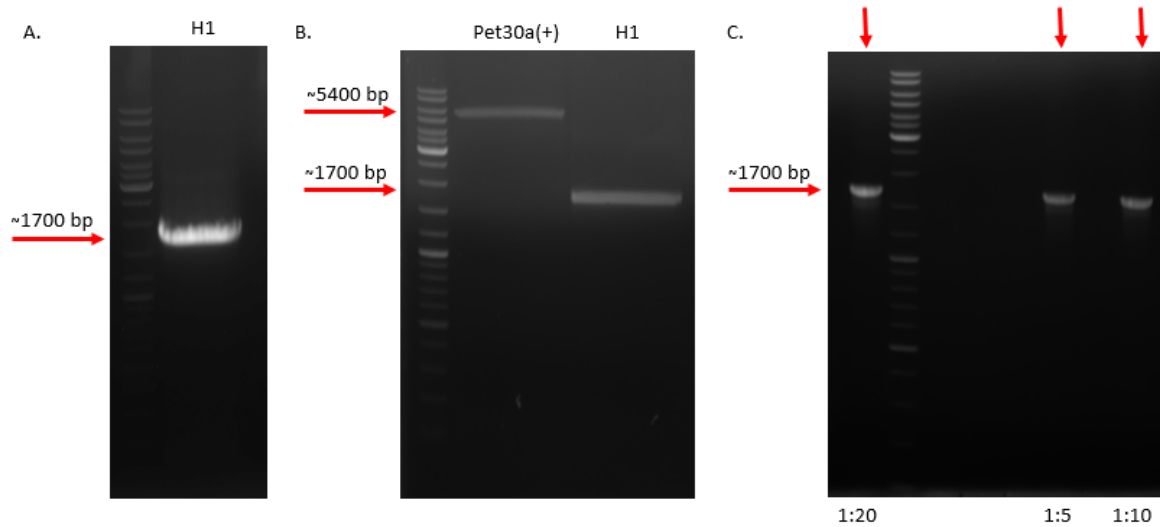


Figure 5.11. Cloning of H1 into pet30a(+) bacterial vector. A. PCR amplification of H1 coding sequence. B. Restriction products of insert sequence(H1) and vector plasmid (pet30a(+)-H5). C. Colony PCR for selected colonies. Red arrows shows positive colonies that gave the correct size after colony PCR reaction.

## 5.7. EG7-OVA Tumor Model

EG7-OVA is one mostly used thymoma model for C57BL/6 mice. This cell line was constitutively expressing chicken antigen ovalbumin when G418 treats the cells and these are useful for study antigen-specific anti-tumor vaccines.

### 5.7.1. Expression of OVA in EG7-OVA Thymoma Cell Line

8-12 weeks old C57BL/6 mice have used this study.  $2.5 \times 10^6$  EG7-OVA cells were inoculated at the right dorsal flank of mice after the expression of OVA for EG7-OVA cells were confirmed by western blot analysis as seen in Figure 5.12. 12-14 days after the inoculation, all tumors reached to a palpable size ( $\geq 50mm^3$ ) then immunization for each mice was performed.

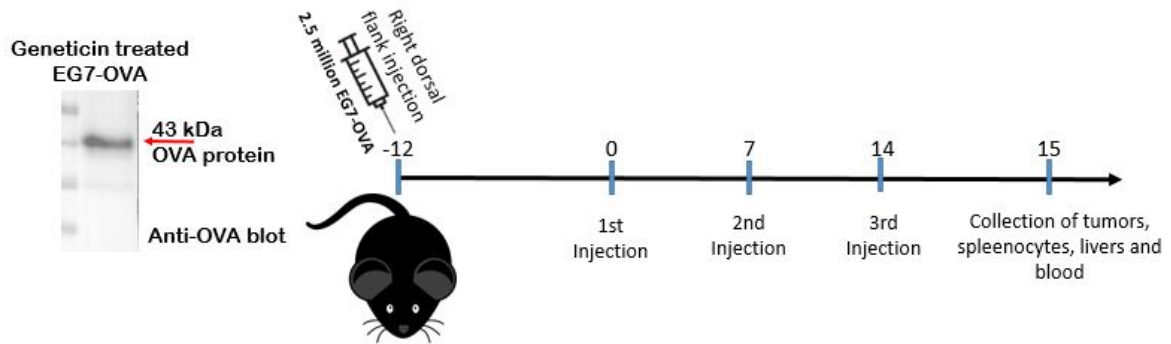


Figure 5.12. Vaccination timeline for immunization of mice that had EG7-OVA tumors.

There were 4 groups including PBS, OVA, tOVA-ASC and mCh-ASC+OVA experimental groups. Only PBS, only 100  $\mu$ g of OVA, only 100  $\mu$ g of tOVA-ASC and 100  $\mu$ g of mCh-ASC and 100  $\mu$ g of OVA together were injected intraperitoneally to each group of mice. PBS was a negative control group because both OVA and ASC specks were suspended in PBS. Also, we tried to understand whether ASC specks have adjuvant property or not. So, we performed only OVA injections to see only OVA immunization was or not enough for a reduction in tumor size. Also, mCh-ASC with OVA group was a model to see an adjuvant property of ASC specks comparing to OVA group. In this experiment, tOVA-ASC group was the model group to see the carrier ability of ASC specks as antigen-specific anti-tumor vaccine.

### 5.7.2. Progression of Tumors After Intraperitoneal Injections of tOVA-ASC Specks

14 days after inoculation of EG7-OVA cells, tumor formation reached to palpable size to start immunizations. As seen in Figure 5.13, 7 days after the first immunization, although PBS, OVA and mCh-ASC+OVA groups had an increase in tumor size, tOVA-ASC specks immunized mice had almost no progression in tumor sizes. Then, second doses were injected to each group. 7 days after the second immunization, 1 of mCh-ASC+OVA immunized mice and 3 of tOVA-ASC immunized mice had completely

eradicated tumors. Only OVA immunized mice, 1 of them remained at the same size after 2 injections. 1 of mCh-ASC+OVA immunized mice stayed stable tumor size after 2 injections. After last immunization, all animals were sacrificed. Lastly, 1 of tOVA-ASC immunized mice had grown pattern after the injection. We tried to understand both carrier and adjuvant properties of ASC specks and this result showed that carrier ability of ASC specks much more powerful tool to eradicate tumors and adjuvant modality of ASC specks could be investigated further.

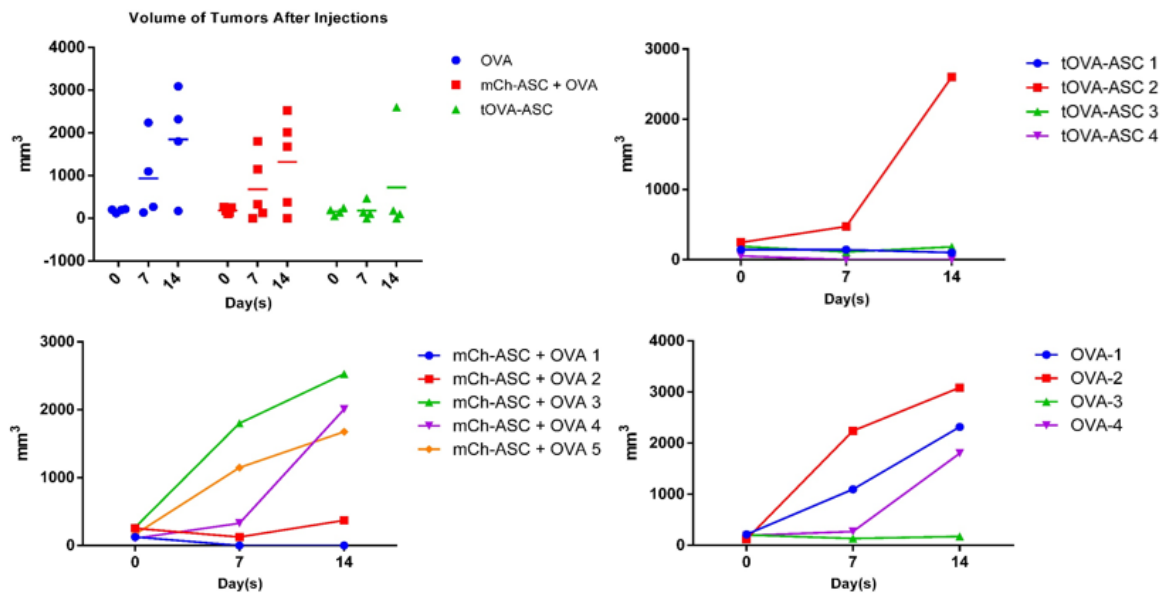


Figure 5.13. Volume of tumors after immunizations. After immunizations tOVA-ASC group had the highest anti-tumor activity comparing to other groups. mCh-ASC+OVA group gave the discussible result because 1 of them had completely eradicated tumor after immunizations. Each dots represents each mice(n=4).

### 5.7.3. OVA Specific IgG Response Against tOVA-ASC Specks

As an anti-tumor therapy, antibodies have been in clinical trials for targeting the tumor cells. These antibodies bind to antigens on tumor cells and this situation induces direct cytotoxicity or antibody promoted phagocytosis, resulting in the processing of antigens and presentation via MHC Class I or II molecules on APCs. This circumstance

induces anti-tumor immunity of host by either the production of the tumor-directed antibodies and/or cytotoxic T cells in the host. Anti-tumor effect of tOVA-ASC specks was observed so, this anti-tumor activity could be caused by antibody promoted anti-tumor immunity. To show antigen-specific antibody production in host, we performed ELISA to see OVA-specific IgG response against EG7-OVA tumors.

As seen in Figure 5.14, PBS was our negative control group, and comparing to other groups with PBS, the increase in the OVA-specific IgG levels were obtained for OVA, mCh-ASC+OVA and tOVA-ASC groups. When OVA including groups were compared to each other, there was a significant higher IgG response for mCh-ASC+OVA group comparing to the OVA group. Also, there was a significant higher IgG response for tOVA-ASC group comparing to the OVA group. This showed that ASC specks could have anti-tumor activity by boosting the immune system as an adjuvant and by carrying antigens to induce anti-tumor immunity in the host.

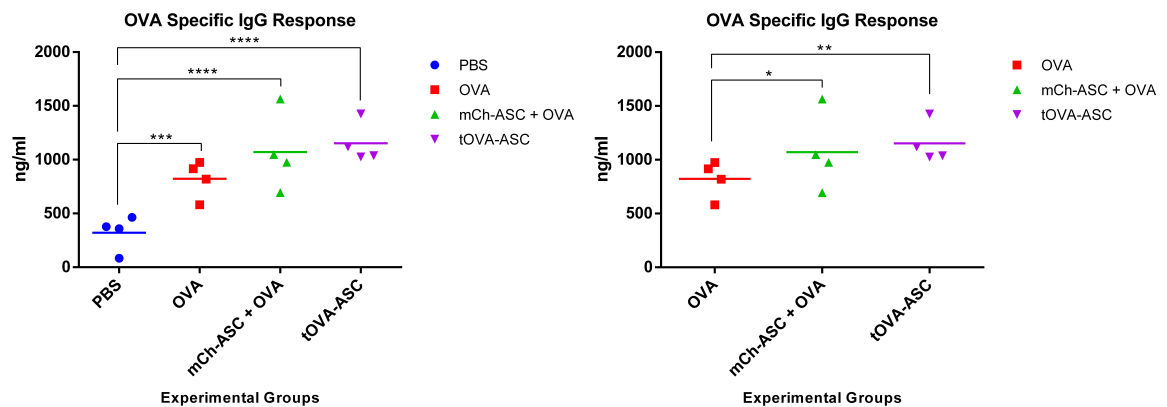


Figure 5.14. OVA specific IgG response for experimental groups of EG7-OVA model.

OVA specific IgG response was the highest for tOVA-ASC immunized mice. Comparing to OVA group mCh-ASC+OVA group had higher OVA specific IgG levels. There was significant increase in the level of IgG for comparison of OVA, mCh-ASC+OVA and tOVA-ASC groups to PBS group. (n=4 for each group)

## 6. DISCUSSION

ASC proteins are oligomerized and combine to each other to form a large structure called ASC speck. It has a unique nature for the formation of micrometer size stable structure. Not only ASC has an adaptor role in the formation of the inflammasome complex but also studies showed that cytokine processing dependent on caspase-1 activation occurs through the formation of ASC specks. ASC specks are formed via aggregation of ASC proteins together and have a compact shape speck-like structure that is micrometer in size. These ASC specks are formed rapidly that takes a few minutes. These ASC specks can be secreted to the outside of the cell to accelerate the recruitment of immune cells. Thanks to highly specific interactions between PYD and CARD domains, oligomerization of ASC proteins and the formation of the ASC speck process is completed rapidly and one cell is able to form only one ASC speck. The formation of ASC speck in rapid progress provides a quick immune response as the first line of defense of the immune system.

It has been previously reported that ASC protein is involved in antigen presentation via both inflammasome-dependent and independent mechanisms. The mechanism of mostly used adjuvant "Alum" depends on the ASC protein in the NLRP3 inflammasome pathway. There are several health concerns about the usage of Alum as an adjuvant in the vaccines. This leads to ongoing research in order to find new safer adjuvants for vaccine technology.

Currently, studies are conducted for the development of particulate adjuvants which may target APCs and they might be able to activate the immune system efficiently to produce antibodies against a pathogen through a single injection. Dendritic cells and macrophages are major types of APCs in the immune system. Thus, we wanted to show that ASC specks can be engulfed by macrophages and lead to the activation of certain mechanisms as adjuvant characteristics.

In this M.S. thesis, we aimed to investigate ASC specks to be used as carriers and

adjuvant in the vaccine technology. Since these ASC specks have unique properties in contrast to other aggresome-like structures, using ASC specks may be an advantage for clinical trials. In our studies, we showed that ASC specks could co-aggregate with cytoplasmic hydrophobic proteins. Also, artificial loading on ASC specks makes them possible as carriers. This information gave an idea that antigens from pathogens might also co-aggregate on ASC specks. This could be a new way of antigen presentation which makes informative for the development of new kind of vaccines. Also, our studies showed that these ASC specks were stable at 37 °C for over 30 days and this gave an advantage over many vaccines because of no need for refrigeration which makes transportation of them to overseas without consideration of their potency. In addition to that these ASC specks could be stay stable and potent inside of the body because our studies showed that after C57BL/6 mice were vaccinated with mCherry-ASC specks, these were found in the spleens of mice after immunization. So, this strong resistance could be useful for no need of repeated injections regularly to boost the immune system.

There are many mechanisms that show actions of adjuvants including enhancement of uptake of antigens by APCs. One of our aim for this project is the investigation of these ASC specks as adjuvants. Therefore, THP-1 monocytes were differentiated to macrophages by using PMA and ASC specks(mCherry-ASC) were given to macrophages. This experiment showed that macrophages engulfed these mCherry-ASC specks. Moreover, activation of the inflammasome is one of the action mechanism for adjuvants. To check whether ASC specks can have an influence on the inflammation pathway, levels of secreted cytokines IL-1beta and TNF-alpha were checked. After macrophages treated with ASC specks, and these ASC specks were engulfed by macrophages, supernatants from each sample were collected to measure the amount of secreted cytokines. We showed that after the engulfment of ASC specks, both IL-1beta and TNF-alpha levels were significantly increased in comparison with non-treated macrophages. These results showed that ASC specks had also action mechanisms as adjuvants including a role in inflammation and antigen uptake by APCs.

One of our aim about this project is to develop a novel vaccine against H5N1 influenza using ASC specks as carriers to increase antigen presentation and adjuvants

to enhance the response of the immune system. ASC specks loaded with antigens(tH5) were used to immunize mice in order to show the effects of ASC specks as carriers/adjuvants. For that purpose, firstly tH5-ASC expressing eukaryotic plasmid vector was constructed. In the cell culture, HEK293FT cells were co-transfected with plasmids expressing mCherry-ASC and tH5-ASC proteins. After 48 hours of transfection, each one ASC speck per cell was collected and purified successfully. To show their effectiveness as a vaccine against H5N1 influenza, C57BL/6 mice were vaccinated with these specks and their blood samples were collected to check their H5 specific IgG levels. In addition that, before each vaccination, weights of mice were measured and their current health conditions were checked. During the process of vaccination, each mice had no negative effect on their health situations. After taking their blood samples, the plasma of each sample was collected and used for the ELISA experiment to show the H5 specific IgG levels. Therefore, ELISA plates were covered with H5 protein and samples were compared to their H5 specific IgG levels.

There were four different groups including PBS, mCherry-ASC, Alum+tH5 and mCherry-ASC+tH5-ASC for the study about vaccine development against H5N1 influenza. Alum is one of known adjuvant and we used this adjuvant especially to see whether antigen-loaded ASC specks also had an adjuvant-like property in addition to the property as carriers for antigens. We showed that tH5-ASC vaccinated mice had higher H5 specific IgG levels in contrast to other experimental groups including tH5+Alum for each vaccination timeline. After first immunization, there was an increase in H5 specific IgG responses for mCh-ASC+tH5-ASC and Alum+tH5 groups although there was a basal level of response for control groups including PBS as a negative control group and mCherry-ASC as a control group for antigen specificity. After the second immunization, almost the same pattern as results of the first immunization was obtained. After the third(last) immunization, there were significant results that supported our proposed hypothesis for the development of a prototype vaccine against H5N1. Mice immunized with the tH5-ASC group had the highest H5 specific IgG levels among others. There was almost 8 times more response in contrast to PBS and mCherry-ASC groups and there was almost 3 times more response in contrast to Alum+tH5 group. The level of H5 specific IgG response for Alum+tH5 group was still

higher than PBS and mCherry-ASC groups as expected. As a result, Alum with tH5 group had lower H5 specific IgG levels comparing to only tH5-ASC group had. So, we concluded that tH5-ASC specks could increase H5 specific IgG response significantly. This result confirmed that ASC specks could be a novel prototype influenza vaccine against H5N1 using the antigen carrier ability of ASC specks and the adjuvant ability for better immune response.

Our last aim for this project is to develop novel cancer immunotherapy using ASC specks. As previously mentioned, we showed that ASC specks could be loaded with antigens and they stayed stable and potent that provided an advantage over many proposed vaccines. EG7-OVA cell line was used for this study. EG7-OVA cells are used as a model system to study MHC Class I restricted immune responses of cytotoxic T cells in mice. This cell line is expressing OVA antigen consistently when treated with G418. In our lab, tOVA-ASC expressing plasmid was successfully constructed. To see the effect of tOVA-ASC specks on tumor progression, mice with EG7-OVA tumor were immunized with tOVA-ASC specks and tumor progression was monitored. Before the inoculation of EG7-OVA cells, western blot analysis was performed to see OVA expression from the cells. After the inoculation of tumor cells, mice had a palpable size of tumor 2 weeks after the inoculation.

There were 4 experimental groups including PBS, OVA, mCh-ASC+OVA and tOVA-ASC to study cancer immunotherapy using ASC specks. PBS group was a negative control group and we expected to see huge tumor progression for this group. We used the only OVA to observe whether the antigen was enough to occur efficient response to slow or stop the progression of tumors. mCh-ASC+OVA group was used to indicate an adjuvant property of ASC specks with antigen separately. The main group of our proposed cancer immunotherapy was the tOVA-ASC group to monitor the antigen presentation on ASC specks to the immune system. After the first immunization, there were significant differences between groups. Mice vaccinated with PBS had the highest volumes of tumors for each mice. OVA-immunized mice had slightly decreased tumor volumes comparing to the PBS group. Mice immunized with the mCherry-ASC+OVA group had more decrease in sizes of tumors in comparison

with the OVA group. As an expected result, mice immunized with the tOVA-ASC group had the lowest tumor sizes in contrast to other experimental groups. The differences between volumes of tumors for each group clearly appeared after the second immunization. All mice for the PBS group had the highest volume of tumors among other experimental groups. All mice for tOVA-ASC group had the lowest volume of tumors among the others because there was the complete eradication of tumors for some mice in this group. It was also interesting to show a complete eradication of tumor for the mCh-ASC+OVA group. Also, there was a mouse had a lower volume of tumor size in the OVA group but there was no eradication like mCh-ASC+OVA and tOVA-ASC groups had. As a result in general, OVA-immunized mice had a lower response against tumor progression, their tumors were grown continuously. Although mCh-ASC+OVA group generally showed an increase in tumor growth, there was one mouse that had no tumor after the second immunization. This result gave an idea about these ASC specks could act as an adjuvant for OVA presentation. Especially, we observed expected results for tOVA-ASC group. Some of tOVA-ASC immunized mice had no tumor after the second vaccination. Generally, the tOVA-ASC group had the lowest tumor progression among others. This tumor progression result confirmed that ASC specks could be novel cancer immunotherapy for antigen-specific tumors using the antigen carrier ability of ASC specks and there could be an adjuvant effect of ASC specks on cancer immunotherapy.

15 days after the immunizations for the study about cancer immunotherapy, all mice sacrificed and their tumors and blood samples were collected for further studies. Their plasma from blood samples was collected and used for ELISA experiment. After the several immunizations, we expected to observe an increase in OVA-specific IgG responses for OVA containing groups except for only PBS group. Therefore, ELISA plates were covered with OVA protein and samples were compared to their OVA-specific IgG levels. As a result of vaccinations, mice immunized with OVA had higher levels of OVA-specific IgG in contrast to PBS group had. Also, mCh-ASC+OVA and tOVA-ASC groups had higher levels of OVA-specific IgG responses in comparison with the PBS group. When we compared OVA, mCh-ASC+OVA and tOVA-ASC groups with each other, we observed a significant difference between OVA and mCh-ASC+OVA

groups and OVA and tOVA-ASC groups. Although OVA-immunized mice had higher IgG responses in contrast to PBS group had, their IgG levels were found significantly lower than mCh-ASC+OVA immunized mice had. Moreover, mice immunized with tOVA-ASC had significantly higher OVA-specific IgG levels in contrast to mice immunized with OVA had. These results showed that antigens carried on ASC specks caused more OVA-specific IgG response. This result supported our proposed cancer immunotherapy model using ASC specks because these results confirmed that more IgG responses gave more decrease in tumor growth and led to slower tumor progression.

In conclusion, our studies showed that ASC specks can be used for delivery of antigens to APCs and are able to boost the immune system and provide the production of the large quantity of antibodies against an antigen. Although we did not use any adjuvant with OVA-loaded ASC specks, the antibody production against OVA antigen was sufficient for the induction of immune response compared to other experimental groups. Also, our studies indicated that ASC specks can be used as antigen delivery vehicle against H5N1 influenza. After the third injection for the immunization, tH5-ASC immunized mice had higher H5 specific IgG responses compared to other experimental groups. Humoral immune responses against antigen-loaded ASC specks also support that these ASC specks can be used as a novel carrier/adjuvant in vaccine technology. Of course, the challenge experiments should be done with the pathogen after antigen loaded-ASC speck immunization to evaluate initial efficacy of the purposed vaccine before large-scale field tests are conducted. Besides, we showed that immunization with tOVA-ASC specks provides not only significant decrease in EG7-OVA tumors but also complete eradication of the tumors. It would be interesting to see which type of immune cells are recruited to the place of tumor and which mechanisms are involved in the slowing down tumor progression. It would be also interesting to show progression of tumors after the complete eradication of EG7-OVA tumors for tOVA-ASC immunized mice without further injections. Our findings support that ASC specks can be a novel therapeutic vaccine by carrier/adjuvant modality for the development of further vaccines.

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APPENDIX A: PLASMID MAPS

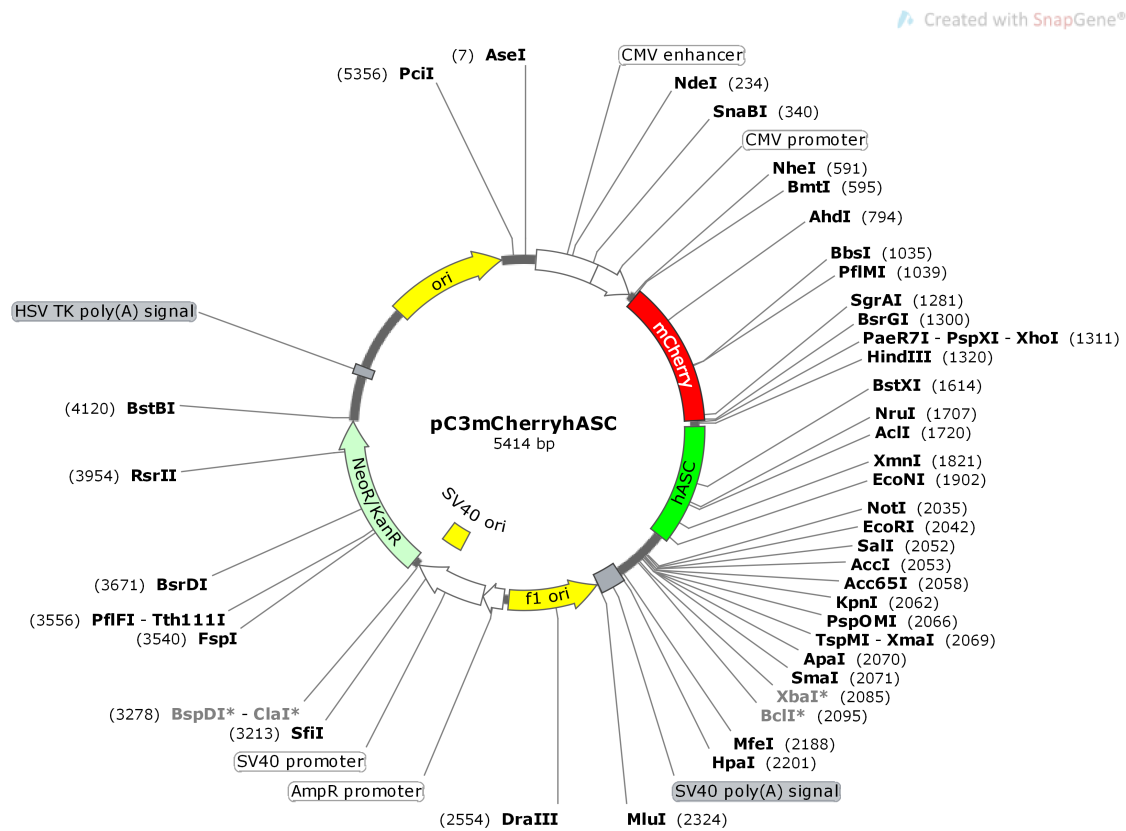


Figure A.1. Plasmid map of pC3-mCherry-hASC

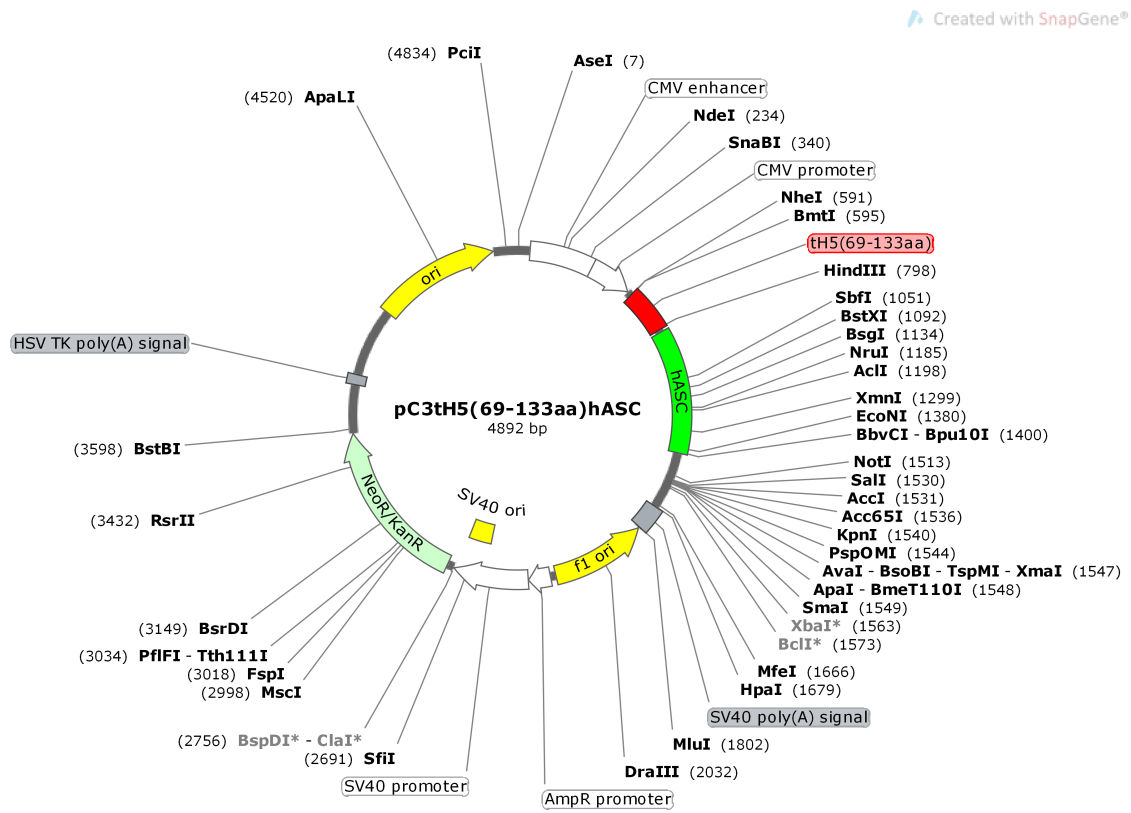


Figure A.2. Plasmid map of pC3-tH5(69-133aa)-hASC

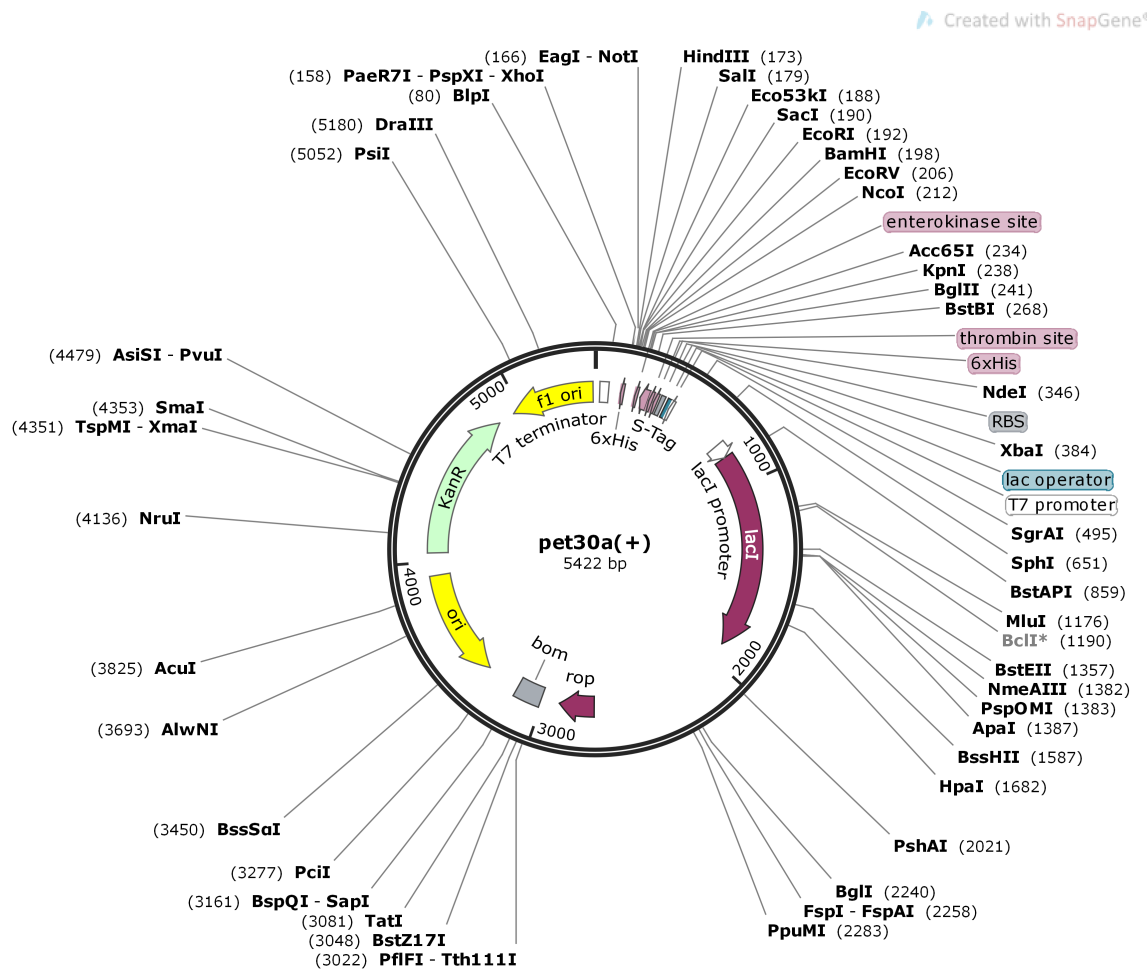


Figure A.3. Plasmid map of pet30a(+)

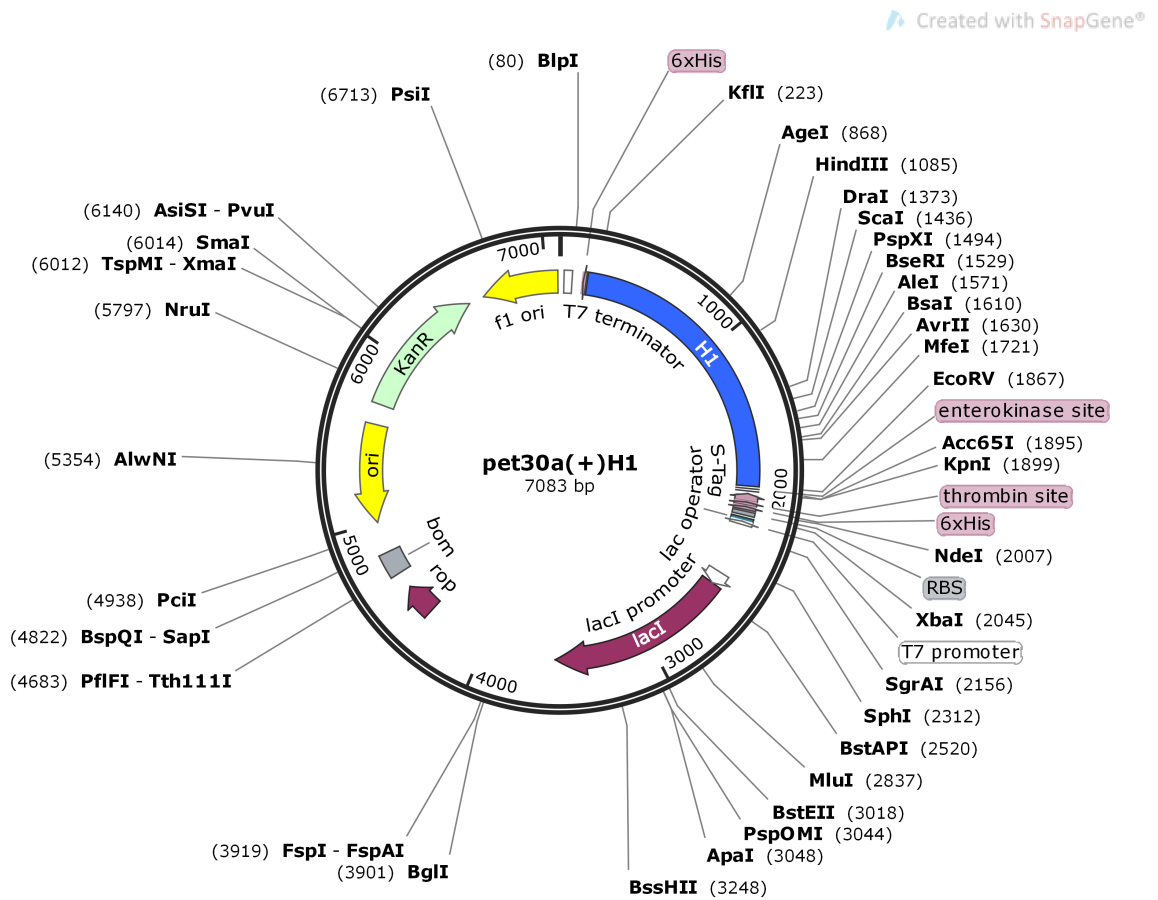


Figure A.4. Plasmid map of pet30a(+)-H1

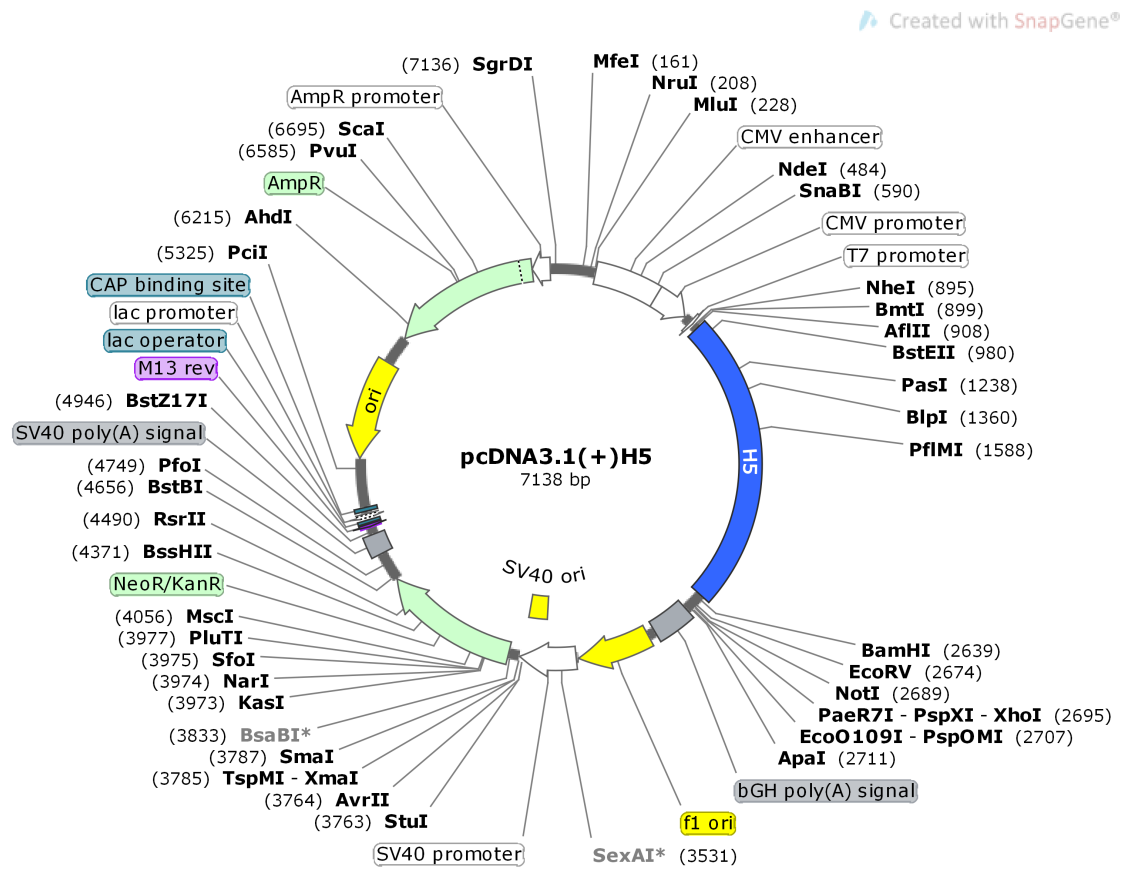


Figure A.5. Plasmid map of pCDNA3.1(+)-H5