INVESTIGATING IMMUNOMODULATOR MECHANISMS OF *ASTRAGALUS* SAPONINS

Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology and Bioengineering

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July, 2018
İZMİR

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ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my advisor Prof. Erdal BEDIR for the continuous support of my master thesis and research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor for my master study. I would also thank my co-advisor Assoc. Prof. Duygu SAG WINGENDER for opening her laboratory and for her knowledge, support and suggestions. I am grateful to Prof. Ali CAGIR for his knowledge, help and suggestions.

Many thanks need to be given to my labmates of Natural Product Chemistry Laboratory and Sağ Laboratory for the sleepless nights we were working together, their help and support all the time.

Finally, I must express my very profound gratitude to my parents for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

ABSTRACT

INVESTIGATING IMMUNOMODULATOR MECHANISMS OF *ASTRAGALUS* SAPONINS

Adjuvants are chemical/biological substances that are used in vaccines to increase immunogenicity of antigens. Astragaloside VII (AST VII), a triterpenoid saponin isolated from *Astragalus* species, stimulates Th1 mediated immune response with antigen specific antibody response. The main goals of this thesis are synthesis of immunologically active analogs of AST VII and identifying immunomodulatory mechanism of actions of AST VII. The impact of AST VII and its synthesized analogs (dicarboxylic AST VII: DC-AST VII and dodecylamine conjugated AST VII: DAC-AST VII) on the cytokine release profile of human whole blood cells (hWB), dendritic cell maturation and subsequently T cell activation were analyzed by using flow cytometry and ELISA. IL-1β and IL-17A cytokines were substantially induced on hWB following treatments of the compounds. The most potent compounds were: DAC-AST VII (3.32 fold) for production of IL-1β, AST VII (5.05 fold) for production of IL-17A. AST VII was more effective than DAC-AST VII (7.52 fold versus 1.34) in IL-1β production in BMDCs (bone marrow derived dendritic cells). The co-stimulation with AST VII and LPS enhanced dendritic cell maturation and activation by upregulating MHC II, CD86 and CD80, as well as IL-12 induction. All compounds were able to activate $CD4^+$ and $CD8^+$ T cells via increasing CD44 expression. Inflammasome activation may have a role in AST VII induced IL-1β secretion, dendritic cell maturation and T cell activation. However, more detailed molecular mechanism studies are warranted to substantiate our findings and to put forward signaling pathways involved.

ÖZET

ASTRAGALUS SAPONİNLERİNİN İMMÜNOMODÜLATÖR MEKANİZMALARININ ARAŞTIRILMASI

Adjuvanlar, antijenlerin immünojenisitesini arttırmak için aşılarda kullanılan kimyasal/ biyolojik maddelerdir. *Astragalus* türlerinden izole edilmiş bir triterpenoid saponin olan Astragaloside VII (AST VII), antijen spesifik antikor yanıt ile Th1 aracılı immün yanıtı uyarmaktadır. Bu tezin ana amaçları AST VII'nin immünolojik olarak aktif yeni analoglarının sentezi ve AST VII için olası immünomodülatör etki mekanizmalarının tanımlanmasıdır. AST VII ve son zamanlarda sentezlenen analoglarının (dikarboksilik AST VII: DC-AST VII ve dodesilamin konjuge AST VII: DAC-AST VII) insan tam kandaki sitokin salım profili, dendritik hücre olgunlaşması ve ardından T hücre aktivasyonun etkisi akım sitometrisi ve ELISA kullanılarak analiz edildi. IL-1β ve IL-17A sitokinleri hWB'ta moleküllerin uygulamalarına takiben önemli derecede indüklenmiştir. En potent moleküller şunlardır: IL-1β üretimi için DAC-AST VII (3.32 kat) ve IL-17A üretimi için AST VII (5.05 kat). AST VII, BMDC'lerde (kemik iliği türevli dendritik hücreler) IL-1β üretiminde DAC-AST VII'ye göre (7.52 kata karşılık 1.34) daha efektiftir. AST VII'nin LPS ile birlikte ko-stimülasyonu MHC II, CD86 ve CD80'i up-regüle ederek ve aynı zamanda IL-12'yi salgılayarak dendritik hücre olgunlaşmasını ve aktivasyonunu arttırdı. Tüm bileşikler CD44 ekspresyonunu artırarak $CD4^+$ ve $CD8^+$ T hücrelerini aktive edebildi. İnflamazom aktivasyonu, AST VII ile uyarılan IL-1β sekresyonu, dendritik hücre olgunlaşması ve T hücresi aktivasyonunda rol oynayabilir. Bununla birlikte, bulgularımızı kanıtlamak ve sinyal yollarını ortaya koymak için daha ayrıntılı moleküler mekanizma çalışmaları gereklidir.

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ABBREVIATIONS

1D-NMR: One-Dimensional Nuclear Magnetic Resonance

2D-NMR: Two-Dimensional Nuclear Magnetic Resonance

a-Gal: a-galactosylceramide

ALRs: AIM2-like receptors

Alum: Aluminum sulfate

APC: Antigen Presenting Cells

ASC: Apoptosis-related speck-like protein containing a caspase recruitment domain

AST II: Astragaloside II

AST IV: Astragaloside IV

AST VII: Astragaloside VII

BMDCs: Bone marrow derived dendritic cells

BMDMs: Bone marrow derived macrophages

CARD: Caspase activation and recruitment domain

CC: Column Chromatography

CCR7: Chemokine receptor 7

$CHCl_3$: Chloroform

CLRs: C-type lectin receptors

DAC-AST VII: Dodecylamine Conjugated AST VII

DAMPs: Damage Associated Molecular Patterns

DCs: Dendritic cells

DC-AST VII: Dicarboxylic AST VII

DPBS: Dulbecco's Phosphate Buffer Saline

EtOAc: Ethyl acetate

FACS: Fluorescence Activated Cell Sorting

FBS: Fetal Bovine Serum

FDCs: Follicular dendritic cells

GM-CSF: Granulocyte macrophage colony stimulating factor

HSQC: Heteronuclear Single Quantum Coherence

HMBC: Heteronuclear Multiple Bond Coherence

hWB: Human whole blood

H_2O: Water

ICAM-1: Intercellular Adhesion Molecule 1

LPS: Lipopolysaccharide

M-CSF: Macrophage colony stimulating factor

MeOH: Methanol

MCP-1: Monocyte Chemoattractant Protein-1

MHC: Major Histocompatibility Complex

MIF: Macrophage Migration Inhibitory Factor

MLR: Mixed Leukocyte Reaction

MPL: Monophosphoryl lipid A

MS: Mass Spectroscopy

MSU: monosodium urate

NF-κB: Nuclear Factor Kappa B

NLRs: Nucleotide binding domain leucine-rich repeat containing receptors

NMR: Nuclear Magnetic Resonance

OVA: Ovalbumin

PAMPs: Pathogen Associated Molecular Patterns TLC: Thin Layer Chromatography

PRRs: Pathogen Recognition Receptors

rcf: relative centrifugation force

RLRs: RIG-like receptors

Syk: spleen tyrosine kinase

TCR: T Cell Receptor

Tfh: T follicular helper

Th: T helper

TLR: Toll Like Receptor

VACM-1: Vascular cell adhesion protein 1

WB:Whole Blood

CHAPTER 1

INTRODUCTION

Vaccination is one of the best strategies to induce protective immunity against infectious diseases and cancer. Although discovering a safe and effective antigen has been the main target of vaccinology, the development of an adjuvant (a term come from the Latin word “adjuvare” “to help”) is also gaining equal importance in recent years. Inactivated antigens, recombinant or subunit vaccines, which contain the purified part of the antigen, are favored because of their safer profiles, but their poor immunogenicities require an adjuvant to increase the immune response.

Adjuvants are synthetic or natural substances, widely used in vaccines. The adjuvant concept was first revealed by Gaston Ramon at the Pasteur Institute in 1920 with the observation of high specific antibody response in horse developing abscesses at the injection site.[1] After then, Glenny et al., reported that toxoid precipitated with aluminum potassium sulfate (potassium alum) induced higher antibody response than toxoid alone. Since then, potassium alum has been used as a licensed adjuvant for human vaccines.[2] Besides enhancing immunogenicity of the antigens, adjuvants used in the vaccines in an attempt to enable more rapid immune response with broad antibody and effective T cell response, spare dose of antigen, reduce the number of immunizations, facilitate bioavailability in children, the elderly, and immunocompromised people.[3,4]

Adjuvant selection should be made upon targeted antigen and desired immune response because of each adjuvant-antigen combination have their unique requirements. Although induction of antibody response is enough for the protection against some types of pathogens (*viz.* toxins), strong cellular immunity is required to fight against viral infections and cancer. Therefore, studies for developing vaccine adjuvants/ adjuvant systems have been proceed by pre-clinical and clinical studies.[5,6]

1.1. Innate and Adaptive Immunity

The body respond to new pathogens in three phases: innate phase, early induced immune response and adaptive immune response. Innate phase includes mucosal barriers and secretions, blood, extracellular fluids kill the pathogen or restrict its effects. Early innate immune cells can be activated by recognizing the extracellular and intracellular infectious agents through pathogen associated molecular patterns (PAMPs) through germLine coded pathogen recognition receptors (PRRs), damage associated molecular patterns (DAMPs) and eliminate the infection.[7,8] DAMPs are important for the elimination of pathogens; however, they sometimes cause autoimmune and chronic inflammatory diseases. DAMPs include sugars, lipids, nucleic acids and different metabolites, and some of them can also be recognized by PAMP.[9] PRRs are classified as Toll like receptors (TLR), C-type lectin receptor (CLRs), nucleotide binding domain leucine-rich repeat-containing receptors (NLRs), RIG-like receptors (RLRs) and AIM2-like receptors (ALRs). TLR and CLR are found on the cell surface or endocytic compartments so that these receptors survey functional microbial ligands in the extracellular environment and within endosomes. NLRs, RLRs and ALRs are found in the cytoplasm for interaction with intracellular pathogens.[10]

If an infectious agent can cross these innate lines, adaptive immune response, the third phase, is activated with expansion of antigen specific lymphocytes and formation of memory cells. The adaptive immune response is slower but more flexible compared to the innate immune response, and able to produce immunological memory. T and B cells undergo a recombination of antigen receptor genes to create a novel and unique antigen receptor, capable of recognizing specific antigen unlike innate immune cells, which are used to fix repertoire.

The bridge between innate and adaptive immune responses is dendritic cells (DCs). Dendritic cells differentiate from bone marrow and enter tissues in immature form. In the immature form, DCs can efficiently phagocytose antigens. However, following internalization of the antigen, DCs undergo a maturation process through the upregulation of MHC II, CD86, CD80 co-stimulatory molecules, and are drained to the lymph nodes to activate naive T cells and B cells.

Three signals are necessary to activate naive T cells: i) interaction of the T cell receptor with MHC class molecules on the dendritic cells for providing antigen

specificity, ii) co-stimulation of CD28 or CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) on the T cell with CD86, CD80 co-stimulatory molecules to activate/ inhibit the response for determining to respond to the stimulus or not iii) cytokine secretion for functional polarizations.

After activation, T cells proliferate and differentiate to pathogen specific $CD8^+$ T cells (cytotoxic T cells) and $CD4^+$ T cells (T helper cells). $CD8^+$ T cells enter the circulation to arrive at the inflamed tissue and kill the infected cells with perforin and granzymes. $CD4^+$ T cells activate B cells to produce pathogen specific antibody response. These antibodies can neutralize the pathogens, enhance phagocytosis via Fc receptor, facilitate complement cascade and lysis of the pathogen.[12]

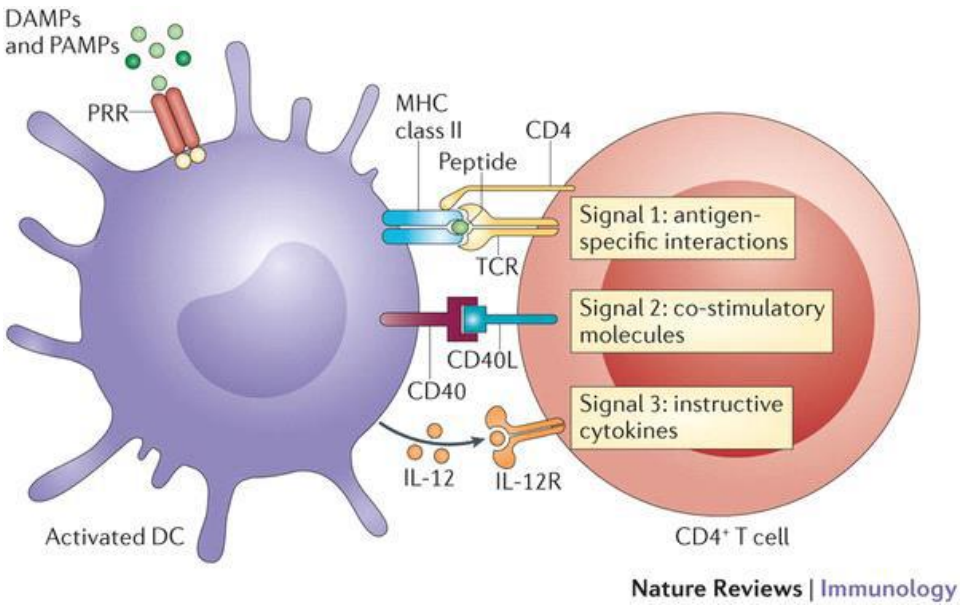


Figure 1.1. Signals required to activate T cells[11]

1.2. History and Classification of Adjuvants

After alum, a number of adjuvants have been developed to induce appropriate immune response as the discovery of timeline shown in Figure 1.2.

Adjuvants can be classified according to their physicochemical properties, origins, and mechanism of actions. Based on the mechanism of action, the adjuvants are

divided into two main groups as delivery systems (particulates) and immunopotentiators (Table 1.1.).

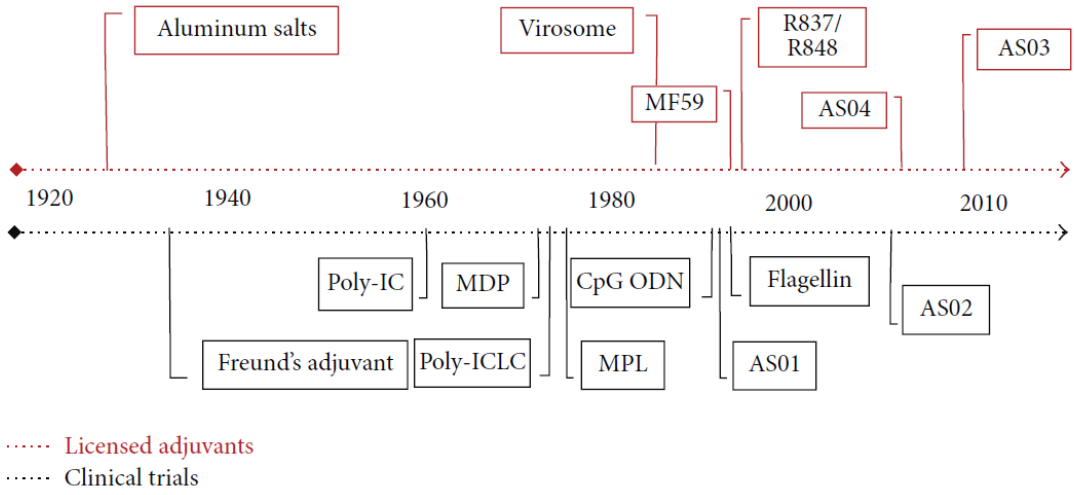


Figure 1.2. Adjuvant discovery timeline[13]

Table 1.1. Classification of adjuvants based on mechanism of actions[13,14]

Delivery Systems	**Immune potentiators**
Mineral salts (Aluminum salts)	Bacterial products (LPS, MPL, flagellin)
Emulsions and surfactant-based formulations (Incomplete Freund's Adjuvant (IFA), MF59, AS02, montanide ISA-51, QS-21)	Toxins and lipids
Particulate delivery vesicles (Micro/nanoparticles, liposomes, virosomes, virus like particles etc.)	Nucleic acids (CpG Oligodeoxynucleotides, Poly(I:C))
	Peptidoglycans (muramyl dipeptide-MDP)
	Saponins (QS-21)
	Small molecules [Imiquimod (R837), Resiquimod (R848)]

1.2.1 Delivery Systems

Delivery systems are formed as carrier to associate the antigen. After the injection of the delivery system, pro-inflammatory cytokines are produced leading to recruitment of the immune cells to site of injection. Therefore, they can activate innate immune cells.

Alum has been used as adjuvant since 1920's. Although depot effect is the main factor for adjuvanticity of alum, recent studies have shown that alum is capable of

stimulating the innate immune response by inflammasome activation without depot effect.[15]

Complete Freund's adjuvant developed in 1930, is water-in-oil emulsion that contain heat inactivated mycobacteria (*Mycobacterium tuberculosis* or others). It is highly powerful, but its reactogenic properties such as formation of granulomas, necrosis at the injection site limits its usage in human vaccines.[16] Incomplete Freund's Adjuvant (IFA), water in oil emulsion without mycobacteria, demonstrates long-lasting antibody response in influenza vaccines by means of slow release of antigen, increasing antigen shelf life and induction of local innate immune response[17]. It induces Th1 driven immune response by producing IL-12, type I IFN, activates antigen presenting cells (APC), and facilitates antigen presentation.[13] MF59®, squalene based water in oil emulsion adjuvant approved for use in influenza vaccine (Fluad™)[18], induces strong antibody response. Activity of MF59 not only comes from depot formation but also induction of macrophages, production of several cytokines and chemokines by dendritic cells, recruitment of the immune cells to injection site and trafficking to draining lymph node.[13]

Virus like particles (VLPs) are composed of viral proteins in the aim of mimicking virus structure and size that are non-replicative and non-invasive. Because of its self-adjuvant properties, they boost B cell proliferation, upregulate genes in class switch recombination and also activate dendritic cells subsequently providing T cell immunity and cross-presentation. There are commercially available vaccines for malaria, HPV (human papilloma virus) and hepatitis B containing VLPs.[19–21] Virosomes, as virus like particles, contain a viral envelope, membrane lipids and glycoproteins, and they act like a delivery system and adjuvant at the same time. They can easily penetrate host plasma membrane due to the presence of hemagglutinin and neuraminidase. Virosomes enhance APC's to induce particle endocytosis, increase co-stimulatory molecules and further $CD4^+/CD8^+$ T cell activation.[22] There are licensed vaccines containing virosomes as adjuvant for influenza and hepatitis A.[23,24]

1.2.2. Immunostimulants

Some adjuvants can interact with PRRs whereas others can be responsible for production of cytokines/chemokines and activate a signaling pathway without PRR

interaction. PRRs can be recognized by different ligands as shown in Table 1.2. Besides PRR ligands, depot effect induced adjuvants such as alum, emulsions, formulations trigger the innate immune response that is important for their adjuvant activity.[25–27]

The type of TLRs and their agonists were illustrated in Figure 1.2. TLRs, are transmembrane glycoproteins, that can recruit and signal through different adaptor molecules such as TIRAP/MyD88, TRAM/TRIF, MAVS, ASC so that different transcriptional outcomes can be obtained.[10]

Table 1.2. Different types of PRRs and their ligands[6]

Type of PRR	**Ligands**
TLRs	Lipids, lipoproteins, nucleic acid, proteins
NLRs	Peptidoglycan, flagellin, toxins, ATP
RLRs	Cytoplasmic RNA
CLRs	Carbohydrates and lipids

Poly IC (polyinosinic:polycytidylic acid) is a synthetic double strand RNA and an agonist of TLR3. It promotes maturation of APC (IL-12 secretion and upregulation of MHC II), antigen processing and activation of the adaptive immune response. Poly IC has been tested in HIV, malaria, dengue and cancer.[13] TLR4 agonist lipopolysaccharide (LPS), which is the outer wall component of gram negative bacteria, has strong immunostimulatory activity triggered by MyD88 and production of TNF-α, but elevated toxicity of LPS limits its usage in human vaccines. Detoxified form of LPS, monophosphoryl lipid A (MPL), was developed exhibiting high levels of antibody and Th1 immune responses by initiating the pathways of TRIF signaling and producing IL-12 and IFN-γ.[12,28] TLR5 agonist flagellin is one of the core components of bacterial flagella from gram-negative and gram-positive bacteria. It enhances TNF-α production, Th1/Th2 mixed responses, activation of inflammasome system as well as boosting high antibody titers.[29–31] Imiquimod (R837) and resiquimod (R848), TLR7/8 agonists, are imidazoquinolines that activate NK cells and dendritic cells, upregulate co-stimulatory molecules, cross-present to $CD8^+$ T cells.[32,33] CpG ODN, synthetic 18-25 oligodenucleotides containing unmethylated cytosine phosphate guadinine (CG) motifs,

is TLR9 agonist and induce antibody and Th1 immune responses.[13] CpG triggers MyD88, IRAK, TRAF-6 signaling pathway, and further activates expression of co-stimulatory molecules (CD86, CD80, CD40) and promotes pro-inflammatory cytokine productions (IL-6, IL-12, TNF-α etc.).

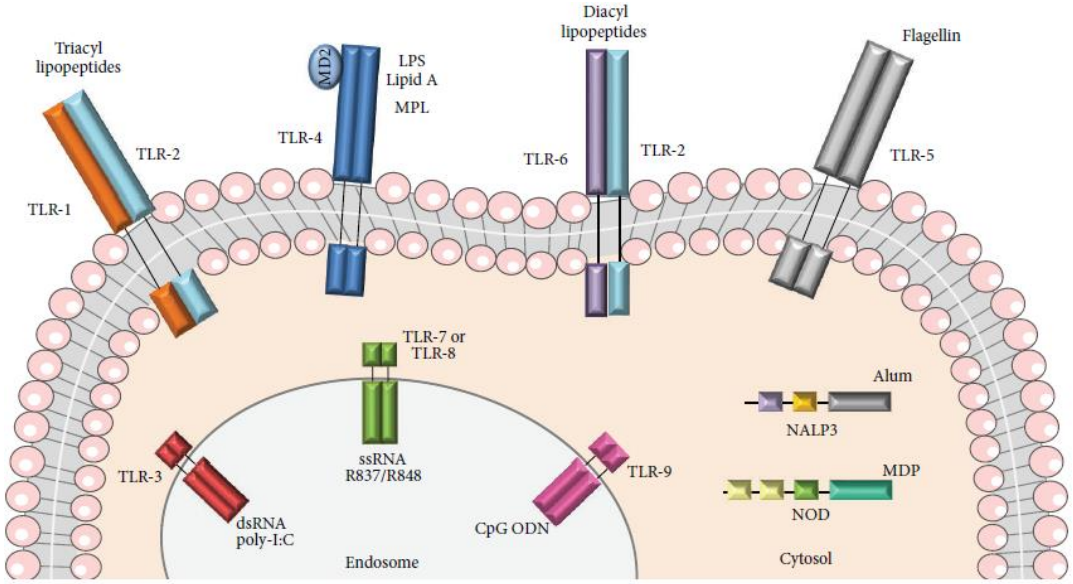


Figure 1.3. Activation of different innate receptors by adjuvants. NALP3: NACHT, LRR and PYD domains-containing protein 3[13]

Besides immunostimulants and delivery systems, TLR agonists are combined in delivery systems to enhance vaccine efficiency via activating different signaling pathways. For that purpose, GSK developed a series of adjuvant systems stated as AS0x. AS01 (MPL, QS-21 and liposome) and AS02 (MPL, QS-21 and squalene emulsion) were tested for malaria vaccines. Based on the results, AS01 was found to induce better antibody response compared to AS02. AS03 is an oil-in water emulsion, composed of α-tocopherol, polysorbate 80 and squalene, used in malaria and influenza vaccines. It showed strong immune response especially in the influenza vaccine. AS04 that contains MPL and aluminum salts are licensed for use in HPV (Cervarix) and HBV (Fendrix®). It is capable of inducing Th1 immune response with the production of IFN-γ and IL-2.[34,35] AS15 adjuvant system containing MPL, QS-21 and CpG was developed for inducing anti-tumor activity in cancer immunotherapeutics such as melanoma and non-small-cell lung cancer.[36]

1.3. Saponins as Immunomodulators

Herbal drugs and their secondary metabolites have been used to treat human diseases since the earliest stages of the civilization. One of the secondary metabolite groups derived from plants or marine organisms and the topic of this thesis, saponins, have a wide range of commercial and industrial application in the pharmaceutical and food industries.

Saponins are characterized based on their skeletons as triterpenoid or steroidal containing one or more sugar moieties.[12] Because of their lipid soluble aglycone part and water-soluble sugar chains, saponins are amphiphilic molecules and widely used as surface active agents. They have exhibited diverse biological activities such as anti-inflammatory, adjuvant, antifungal, antiparasitic, antioxidant, antiobesity, antiviral, diuretic, hemolytic, neuroprotective etc.[12] Saponin based compounds with adjuvant activity and their sources are illustrated in Table 1.3.

Quillaja saponins have caught attention due to their strong immunostimulatory activities in recent years. Quil-A, the aqueous extract of *Quillaja saponaria*, showed induction of humoral and cellular immune responses and used in veterinary vaccines especially in Foot and Mouth Disease. Quil-A is a mixture of QS-7, QS-17, QS-18 and QS-21 fractions. QS-21, purified from Quil-A by reversed phase chromatography, was chosen for further activity studies due to its less toxic profile and more abundancy in the plant than other subfractions. QS-21 has four components in the structure: a central triterpene, branched trisaccharide and linear tetrasaccharide at C-3 and C-28 on the triterpene and hydrolytically labile ester linkage. It demonstrates IgG1/IgG2a balanced antigen specific antibody response along with boosting IFN-γ and IL-2 cytokine titers and effective $CD8^+$ T cell response.[37] Action mechanism of QS-21 states as intercalating cell membrane cholesterols and opening pores, which accelerate the antigen uptake by antigen presenting cells.[38,39] Although QS-21 has high potency as an adjuvant, its toxicity and undesirable hemolytic effect limits its use in human vaccine. To overcome these problems, formulation studies and synthesis of semi-derivative of QS-21 have come up in recent years.

ISCOMs and ISCOMATRIXs, 40 nm sized cage-like particles containing saponin, cholesterol and phospholipid, show humoral, cellular immune response as well as having high stability and decreased level of hemolysis compared to QS-21. As

mentioned before, GSK adjuvant systems containing QS-21 are produced with other immunostimulants (MPL, CpG) in a carrier system to synergize the immune response. AS02 provides strong humoral and T cell mediated immune response and is used for complex disease, requiring effective T cell response, such as tuberculosis, HIV, malaria. AS01 is designed to enhance CD8 T cell response and has been evaluated in malaria, zoster, tuberculosis, HIV vaccines.[36] AS15 is developed for cancer treatments.

One of the analog of QS-21, GPI-0100, is obtained by replacing acyl group at the fucopyranosyl residue with dodecylamide linked glucuronic acid at C-6 at the triterpene C-3. Eleuterio et al., reported that GPI-0100 elicits Th1 immunity and CTL response as QS-21 with less toxicity.[40] Fernandez-Tejada et al., synthesized several QS-21 analogs to investigate structure-function relationship and found that trisaccharide and C-4 aldehyde substituent is not required for adjuvant activity as it proposed in the previous studies that adjuvant active component is C-4 aldehyde substituent, which forms Schiff base with free amino groups on the immune cells.[41] Moreover, active adjuvants accumulate in the injection site and nearest lymph nodes.[42]

Walkowicz et al., derived QS-21 analogs, which retain low toxicity, potent adjuvant activity, high chemical stability, and investigate immunomodulatory properties against MUC1, ganglioside GD3, KLH and OVA in mouse vaccination model. They revealed that junctions between the triterpene and linear oligosaccharide domain have critical role in adjuvant activity of QS-21 derivatives.[43]

1.3.1. *Astragalus* Saponins and Their Immunomodulatory Properties

Astragalus L. (Leguminosae) is a genus distributed broadly throughout the world, located in Asia, North America and Asia.[12] The most common use of *Astragalus* is forage for livestock and wild animals as well as food, medicine, cosmetic applications. *Astragalus membranaceus* is one of the most popular herbal medicine in China. It has been used for the treatment of cold, diarrhea, fatigue and cardiovascular diseases in traditional medicine. In addition, this genus is well known for its pharmacological properties such as immunomodulatory, antioxidant, antitumor, antidiabetic, antiviral, anti-inflammatory etc.

Active constituents are saponins, flavonoids and polysaccharides.[41] Polar fractions or aqueous extract of the *Astragalus* root, which contains polysaccharides,

were investigated for immunomodulatory activity so far. Du et al., demonstrated that *Astragalus* polysaccharide (APS) induces humoral and cellular immune response against hepatitis B subunit vaccine.[44]

Table 1.3. Botanical source and immunomodulatory activities of saponin based compounds[12]

Herbs	**Botanical Source**	**Saponins**	**Adjuvant Effects**
Ginseng	*Panax ginseng*	Crude saponins; Ginsenosides Rg1, Rg2, Rg3, Rb1, Re	-Antibody response against NDV, *Staphylococcus aureus* -Production of IL-1, IL-2, IL-4, IL-10, IFN-γ, TNF-α -Synergistically act with aluminum hydroxide and oil emulsions
Astragalus	*Astragalus membranaceus*	Crude saponins; Triterpene saponins; Astragaloside I, II, IV, VII, brachyosides A-C, cyclocephalo-side I, II	-Increase antibody response to OVA and NDV -Promote IL-1β, IL-2, IL-6, TNF-α -Activate NF-kB
Notoginseng	*Panax notoginseng*	Crude saponins; Ginsenosides, Rh1, Rh4, Rg1, Re, Rb1; Notoginseno-sides; R1, R2, U,K, R4	-Increase antibody response to OVA -Promote production of IL-2, IL-4, IL-5, IFN-γ, TNF-α
Momordica	*Momordica cochinchinensis*	Crude saponins; Momordica saponins I , II	-Promote antibody response to OVA, FMDV, avian influenza virus -Synergistically act with oil emulsion

(cont. on next page)

Table 1.3. (cont.)

Glycyrrhiza	*Glycyrrhiza uralensis*	Crude saponins; Oleanane-type saponins; glycyrrhizin	-Enhance antibody and cellular response against OVA
Achranthes	*Achyrantes bidentata*	Crude saponins	-Enhance antibody and cellular response against OVA
	Platycodon grandiflorum	Crude saponins; Oleanane type saponins; platycodin D, D3	-Enhance antibody and cellular response against OVA

APS enhanced IL-1β, TNF-a, IL-2, IFN-γ, activated mouse macrophages, B cells as well as dendritic cells.[45] However, the saponin part has also revealed immunomodulatory properties. Injection of OVA and *Astragalus* saponin (AS) to mice has shown IgG1 and IgG2b antibody response.[46] Moreover, AS has enhanced T cell transformation, NK cell activity and phagocytosis by macrophages.[37]

In the flora of Turkey, *Astragalus* sp. represents 224 endemic species in a totally 445 species.[47] Bedir et al., isolated 70 cycloartane-type saponins including 5 different aglycones from Turkish *Astragalus* species.[48–53] In the South East Anatolia of Turkey, *Astragalus* root extracts have been used to cure leukemia in traditional medicine. Preliminary studies have focused on cytotoxic properties of the compounds, but there has not been significant activity on tumor cell line following the treatment of the compounds. The hypothesis that the activity could result from immune system has been accepted and further studies have been carried out.

Yeşilada et al., evaluated 13 cycloartane-type and 1 oleanan type triterpene saponins isolated from Turkish *Astragalus* species for cytokine release on human whole blood assay. All saponins have elicited high IL-2 levels with TNF-α, IL-6 and IL-1β. Astragaloside VII (AST VII), named chemically as 3-*O*-β-D-xylopyronosyl-6,25-di-*O*-β-D-glucopyranosyl-20(*R*),24(*S*)-epoxy-3β,6α,16β,25-tetrahydroxycycloartane, a triterpenoid saponins, which has three sugar chains attached to the aglycone at C-3 (xylose), C-6 and C-25 (glucose), has gotten attention due to higher IL-2 production

compared to other saponins. Bedir et al., investigated 19 cycloartane-type tritepene glycosides for human macrophages/monocyte activation by using transcription factor-based bioassay. Only Astragaloside I has the capacity to induce NF-κB (nuclear factor kappa B) and IL-1β mRNA expressions in THP-1 cell line.[54] Nalbantsoy et al., carried out *in vivo* studies by using BSA (Bovine Serum Albumin) and LPS (Lipopolysaccharide) as model antigens. AST VII administration to mice alone has not revealed any effects of cytokine induction on sera. However, AST VII with BSA or LPS have elicited antigen specific IgG, IgG1 and IgG2b antibody response, boosted IFN-γ, IL-2, TGF-β cytokines and splenocyte proliferation.[55,56] Besides cellular and humoral immune response, high solubility in water, slight hemolytic activity at high concentrations, high stability, appropriateness to lyophilization make valuable of AST VII as a potent adjuvant.

1.4. Selection of Adjuvants

Adjuvants should be selected according to desired immune response and targeted antigen. For example, MF59 (squalene-based oil in water emulsion) and ISCOMs (cage like structure containing saponin, phospholipid and cholesterol) could be used to balance Th1/Th2 immune response, whereas administration of Toll like receptor (TLR) 3, 4, 7, 8, 9 agonists lead to a dominant Th1 immune response. Additionally, CAF01 (cationic liposomal vesicle with dimethyldioctadecyl-ammonium and trehalose 6,6-dibehenate)[57] and Freund's Complete Adjuvant (FCA) promote Th1 and Th17 responses.

1.5. Mechanisms of Actions of Adjuvants

Adjuvants could elicit one or more of the following mechanisms to induce immune response: i) depot effect: sustained release of antigen at the injection site, ii) immune cell recruitment to injection site, iii) activation and maturation of APCs (increase in major histocompatibility complex (MHC) class II and co-stimulatory molecules expression), iv) enhanced antigen uptake and presentation to APCs, v)

inflammasome activation, vi) migration to the draining lymph nodes, vii) up-regulation of cytokines and chemokines (Figure 1.4.).[3,58]

i) Depot formation is the oldest and most widely recognized action mechanism of adjuvants. Antigen is slowly released in the injection site, stimulates the immune response prolong period and produces high antibody titers[59]. Alum, oil in water emulsions, micro/nanoparticles, CAF01 have been shown to act by depot effect.

ii) One of the adjuvant action mechanisms is recruitment of immune cells to the injection site. Particulate adjuvants could create a local pro-inflammatory environment for recruitment of the immune cells.[60] MF59 significantly upregulated CCR2 (C-C motif chemokine receptor 2), receptor for CCL2, which is involved in monocyte infiltration. Moreover, MF59 recruited neutrophils, eosinophils, macrophages after intramuscular injection. Alum induced CCL2, neutrophil chemotaxin KC (CXCL1), eosinophil chemotaxin eotaxin (CCL11). AS03 triggered leukocyte- recruiting chemokines CCL2, CCL3, CCL5 and neutrophils, monocytes, eosinophils etc.[58,61]

vi, vii) Several cytokines and chemokines could be produced at the injection site following adjuvant administration. Based on these cytokines/chemokines different immune cells can be recruited and activated, draining lymph nodes to enhance adaptive immunity. Other mechanisms of action (iii, iv, v) were mentioned below with separate sections in detail.

1.5.1. Dendritic Cell Maturation

DCs provide a bridge between innate immunity and adaptive immunity. When dendritic cells receive a signal from the environment, such as interaction of PRR (exposure to Toll like receptor) or damage associated molecular patterns (necrosis, free radicals etc.), inflammatory soluble factors (cytokines), T cell ligands (such as CD40 ligands), DCs switch from immature state to mature state.[62,63]

DCs maturation could be divided into three phases: (i) early maturation (0-6 hours of stimulation) DCs take up antigen and danger signals at the same time, (ii) maturing DC (7-20 hours of stimulation) received the maturation stimulus and takes up additional antigen after initiating their maturation. (iii) fully mature DCs (over 20 hours) cross-presentation of antigen is downregulated but DCs continued to activate naive $CD8^+$ T cell efficiently for cross priming.[64]

DCs have strong phagocytic properties in immature state and phagocytic capacity is decreased following internalization of the antigen. DC maturation is characterized by the increased expression of MHC II molecules, co-stimulatory molecules (CD80, CD86, CD40 etc.), chemokine receptor 7 (CCR7), production of some kind of cytokines (IL-12, interferon α).[4,65–67] DC is presented in the internalized antigen on the MHC molecules to T cell receptor (TCR) and started the subsequent cascade for T cell activation. These MHC- TCR interactions are given signal 1 for the activation, but signal 1 without signal 2 caused stay T cells to stay in unresponsive state (anergy).Signal 2 is the interaction of co-stimulatory molecules on DC and T cells.[4]

Endothelial cells of terminal lymphatic vessels of nonlymphoid tissue upregulate CCR7 ligands in inflammation, thus provide to migrate chemotactic gradient by CCR7 expressed DC to lymph node, where pools of naive T cells, memory T cells and naive B cells are found.[68] DC-T cell interaction in the paracortex region in the lymph node leads to antigen specific T cell activation, expansion, and polarization into T helper (Th) 1, 2, and 17, and T follicular helper (Tfh) cells. Th2 and Tfh cells initiate germinal center reaction where these cells and follicular DCs (FDCs) provide strong activating signals to B cells. The activated B cells produce plasma cells that secrete antigen specific high-affinity Abs.[4]

Adsorbed antigens to alum provide increasing antigen internalization, reducing the rate of degradation.[69] Freund's complete adjuvant, LPS, liposomes, CpG-ODN, MF59, AS04, and a-galactosylceramide (a-GAL) have all shown to induce DC maturation to enhance adaptive immunity PAMP -dependent and independent manner.[70–74]

DCs can be activated by PAMP dependent and PAMP-independent way. As mentioned in the innate and adaptive immunity section, generally PRR ligation started the activation process of DC directly. LPS, TLR 4 agonist, binds to TLR4 and upregulates MHC II, CD80, CD86, CD40 markers related to maturation and activation. The types of adjuvant, binding different PRRs and inducing what type of immune response are listed in Table 1.4. Other than PRR, "danger" signals or inflammatory cytokines induced by DC itself or other immune cells can also activate DC in PAMP-independent manner.[75–77]

Particulate adjuvants such as mineral salts, liposomes, oil in water emulsions, saponins are incapable of DC maturation *in vitro*, but work as an indirect stimulator *in vivo* by targeting stromal cells and other blood type cells as shown in Figure 1.5.

Induction of inflammasome, local recruitment of immune cells and accessory cells play an important role for DCs activation by TLR-independent adjuvants.

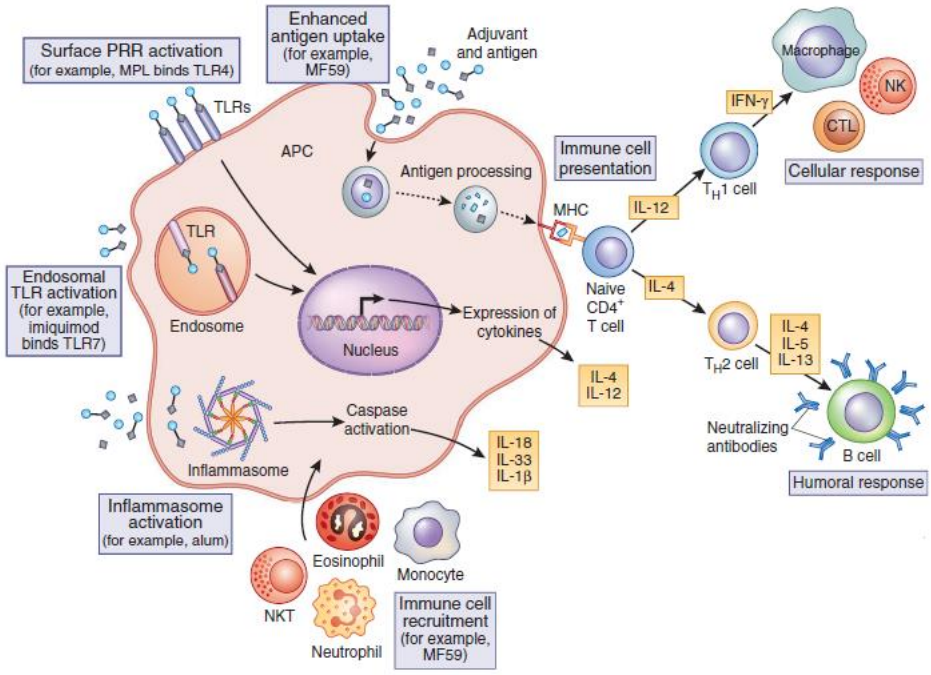


Figure 1.4. Mechanism of actions of adjuvants. A number of mechanisms have been illustrated how adjuvants mediate their activity.[3]

1.5.2. Antigen Processing and Presentation

Efficient antigen presentation is crucial for induction of adaptive immune response. DCs have developed a phagocytic pathway that provides antigen processing and presentation on major histocompatibility complex (MHC) in contrast to macrophages and neutrophils, which destroy antigens in highly degradative compartments by phagocytosis.[78] APCs generally engulfed the antigen from extracellular milieu, placed on MHC II molecules to present $CD4^+$ T cells. Endogenous cytosolic and nuclear antigens are generated by proteasomal degradation, loaded to MHC I molecules to present $CD8^+$ T cells. Exogenous antigens could be loaded to MHC I and presented to $CD8^+$ T cell by means of DC through a process referred to as cross-presentation.[79] Cross presentation could be very effective and necessary for viruses, and do not naturally infect APCs, tumors or tissue specific antigens expressed

by tumor cells, which are not potent for APCs. Moreover, cross presentation gives an alternative pathway for separating antigens in many viruses and tumors, which have the capability to escape immune cells.[80–82]

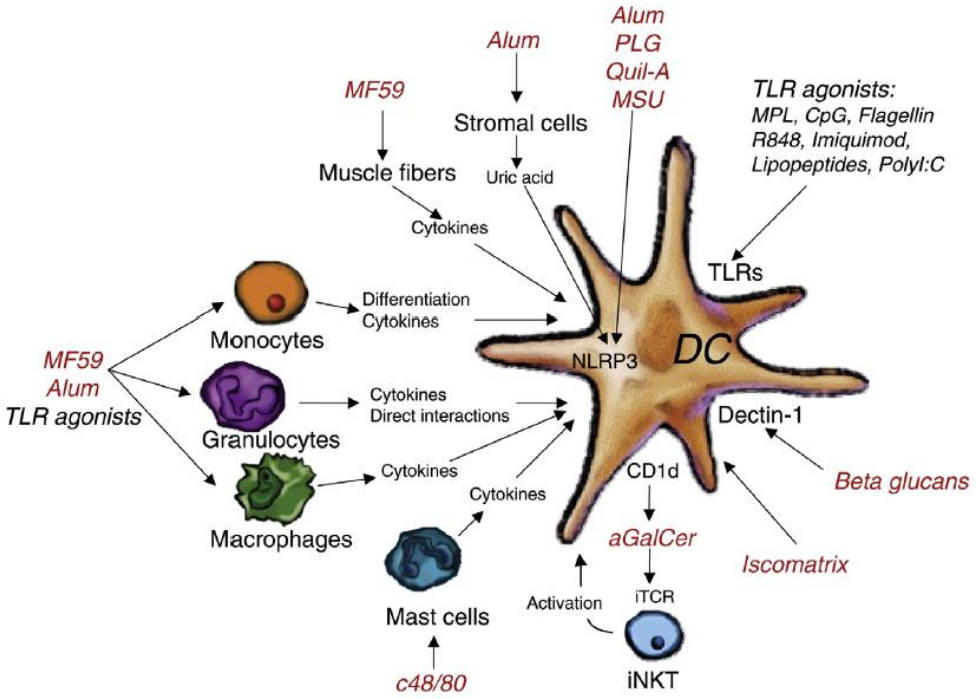


Figure 1.5. Particulate adjuvants action mechanisms. Particulate adjuvants acting on type of immune cells and activation of dendritic cell TLR-independent manner and TLR- dependent manner[58]

Exogenous antigens are cross-presented in two main intracellular pathways referred to as “cytosolic” and “vacuolar” pathways. In the cytosolic pathway, antigens are internalized and degraded through endosomal compartments by enzymatic digestion at acidic pH. After that, antigens are passed to cytosol by an unknown pathway, further degraded in proteasome dependent manner and loaded on MHC I. In vacuolar pathway, cross presentation is proteasome independent. Antigen processing and loading on MHC I are actualized in endocytic compartments.[83]

Different forms of antigens such as soluble proteins, antigen-coated beads, immune complexes, dead cells, can be cross-presented *in vitro* and *in vivo* with danger signals in many circumstances. Engagement of TLR and NLR ligands increases the efficiency of cross priming due to enhancement of T cell co-stimulatory, adhesion molecules, cytokine secretion etc. Burgdorf et al., reported that soluble OVA with low

endotoxin levels cross-presented OVA to T cell less efficiently compared to high endotoxin levels one. In the case of up taking bacteria or dead cells by DC, antigen and danger signals stimulated DC at the same time. After then, TLR and NLR ligands are released and engaged within endosomes and phagosomes. Moreover, they elicited that peptide loading onto MHC I molecules during cross-presentation actualized in endocytic compartments rather than ER.[84,85]

Table 1.4. Activation of PRRs and induction of immune response by vaccine adjuvants[58]

PRRs		Adjuvants	Type of immune response induced
TLRs	TLR1/2	Triacyl lipopeptides Synthetic Pam_3Cys	Th1, Th2, CTL responses
	TLR2/6	Diacyl lipopeptides Pam_2Cys	Th1, Th2, CTL responses
	TLR2	Pam_3Cys	Th1, Th2, CTL responses
	TLR3	Poly I:C	Both Th1 and Th2
	TLR4	LPS, AS04 (MPL)	Th1
	TLR5	Flagellin	Th1 and Th2
	TLR 7	Imiquimod Resiquimod	Th1, $CD8^+$ T cell, CTL responses
	TLR8	Resiquimod	Th1, $CD8^+$ T cell, CTL responses
	TLR9	CpG-ODN	Th1, $CD8^+$ T cells, CTL responses
NLRs	NOD1/NLRC1	DAP	Th1, Th2, Th17
	NOD2/NLRC2	MDP	Th1, Th17
	NLRP1	Toxoids, MDP	Th1
	NLRP3	Alum, MDP, ATP	Th2
	IPAF/NLRC4	Flagellin	Th1 and Th2
	NAIP5	Flagellin	Th1 and Th2
RLRs	RIG-1	DNA vectors	Th1, $CD8^+$ T cells
	MDA5	Poly I:C	Th1, $CD8^+$ T cells
CLRs	Dectin-1	Flagellin, β-glucan/zymosan	Th17
	Mincle	CAF01	Th1, Th17 $CD8^+$ T cells

Alum enhanced the antigen uptake of macrophages and DCs *in vivo* and human peripheral blood mononuclear cells (PBMCs) and DCs *in vitro.*[69,86–90] Alum particles are phagocytosed by macrophages and then phagosomal rupture is observed *in vitro* experiment.[91] Alum phagocytosis is also validated *in vivo* studies inside the macrophages, multinucleated giant cells, MHC II^+ mononuclear cells, DC, and recently within a T helper 1 cell line (THP-1) indicating these APCs actively engulf alum.[92–95] Flach et al. suggested that alum binds DC plasma membrane lipids in cholesterol- and

cellular motility manner. Enhanced antigens are delivered to the cell without alum internalization by lipid sorting with a process called abortive phagocytosis.[90]

Intra-peritoneal injection of OVA and alum lead to maturation of DCs *in vivo* but there are conflicting results of alum induced DCs maturation *in vitro*.[89] Alum and MF59 adjuvants failed to directly activate DCs whereas they have the capability to induce monocyte, macrophages and granulocyte *in vitro*.[96,97] Another study said that alum could activate DCs. These different results following alum administration can be obtained from batch to batch variation of commercial alum adjuvants.

1.5.3. Inflammasome Formation

Inflammasomes are multimeric protein complexes that assemble in the cytosol after sensing PAMPs and DAMPs. Inflammasomes have NOD- like receptor (NLR) sensor molecules such as NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4 etc. ASC (apoptosis-related speck-like protein containing a caspase recruitment domain) contains two death fold domains: one pyrin domain and one caspase activation and recruitment domain (CARD). ASC interacts with inflammasome sensor receptors with its pyrin domain and becomes assembled into a large protein speck. After then, ASC brings the monomer of pro-caspase 1 via its CARD domain and ends with self-cleavage of pro-caspase 1 to active caspase 1. Active caspase 1 proteolytically activates pro-IL-1β and pro-IL-18 and enhances release via non-classically secretion pathway.[98] NLRP-3 inflammasome can be activated by exposure to silica, DAMPs, metabolic stress or pathogen associated molecular patterns (PAMPs) such as bacterial flagellin.[99,100]

TLR ligands or muramyl dipeptide do not directly activate NLRP-3 inflammasome, but they can ensure a priming step for inducing NLRP3 and pro-IL-1β expression through activation of NK-kB. The second signal is triggered by ATP, viral RNA, pore-forming toxins or particulate matters for inflammasome activation that results in the activation of caspase-1 and cleavage of pro-IL-1β to mature IL-1β.[101]

IL-1β and IL-18 are potent pro-inflammatory cytokines that are crucial for host-defense responses to infection, injury, differentiation of Th17 cells and drive IFN-γ mediated Th1 immune response.[102] They are produced by different cell types, but monocyte and macrophages are the ones that are most focused on. Moreover, IL-1 plays

a role in the regulation of DC activation, production of cytokines and differentiation of naive T cells to effector cells.[103]

One of the action mechanisms of alum is inflammasome. *In vitro* studies made by alum showed that inflammasome activation is necessary for alum induced immune response in terms of IL-1β and IL-18 productions. Priming with LPS to upregulate NLPR3 and pro-IL-1β expression is required to evaluate inflammasome activator such as alum *in vitro.*[91,101]

Eisenbarth et al. and Li et al., demonstrated that alum adsorbed OVA antigen injection to NLPR3, ASC, caspase-1 knocked out mice and failed to induce antigen specific immune response.[104,105] Therefore, NLRP3 inflammasome is crucial for alum adjuvanticity and its activity is caspase-1 dependent. However, human serum albumin plus alum injection does not change the antigen-specific IgG production in the NLRP3 deficient mice.[106] Another point of view related to alum's action mechanism is DAMP production. Injection of alum triggers the release of uric acid with tissue irradiation and cell death. Engulfment of alum particle results in lysosomal membrane damage, cathepsin B release and NLPR3 inflammasome activation.[90] Transfer of QS-21 bind in liposome system formulated with cholesterol to cell membrane cholesterol or cholesterol dependent endocytosis in dendritic cell resulted with accumulation of QS-21 in the lysosome. Concentrated QS-21 in lysosome caused pore formation and release of macromolecules and lysosomal cysteine proteases such as cathepsin B to cytosol. Cathepsin B can activate NF-κB and promote the transcription of pro-inflammatory cytokines. Cathepsin B is related to antigen processing and then effects the antigen presentation. QS-21 has degraded protein antigens in the lysosome and the fragments released to cytosol for cross-presentation. In cathepsin-B deficient mice, QS-21 induced $CD8^+$ T cell response lessened that indicating QS-21 and antigens could be displayed in different localizations.[107,108]

Binding of aluminum adjuvants to dendritic cell membrane lipid caused reassortment of the lipids and aggregation of lipid rafts lead to activation of Syk kinase and PI3K (phosphoinositide-3 kinase) pathways. Abortive phagocytosis of antigens but not alum adjuvants resulted in inefficient cross-presentation. In addition, some studies demonstrated that alum can be internalized by macrophages and dendritic cells *in vitro* and *in vivo*. PI3K activation continued with P2X7 receptor-ATP binding, which is required for inflammasome activation by extracellular ATP.[90,109,110] Alum tip binding

with different cell type such as dendritic cell, macrophages and B cell was investigated and only DC showed binding force with alum tip. Alum can interact but seldom enter DC *in vitro* because of incomplete phagocytosis.[90]

1.6. Aims of the Thesis

The discovery of new adjuvants, which demonstrate robust cellular and humoral immune response with low toxicity, have a major role in the field of vaccinology at the point of improving existing vaccines and producing effective vaccines against diseases such as malaria, cancer and HIV. Moreover, understanding the action mechanisms of adjuvants can be led to design and select the best formulation for a vaccine.

The first objective of this thesis was to clarify the mechanism of actions of AST VII in the aim of shedding light into immunomodulatory mechanism of action of tridesmosidic saponins. For that, the effects of AST VII treatment on bone marrow derived dendritic cells, bone marrow derived macrophages, $CD4^+$ T cells and $CD8^+$ T cells would be elucidated in terms of expression of activation and maturation markers and production of cytokines by using flow cytometry and ELISA.

The second objective of this thesis was to obtain semi-synthetic derivatives of AST VII and investigate their effects on the cytokine network for establishing structure-activity relationship. Semi-synthetic derivatives of AST VII would be synthesized through oxidation of primary alcohols to carboxylic acids and subsequently conjugation of free dodecylamines to glucuronic acids via amide bond formation. The compounds would be purified by column chromatography and subsequently the chemical structure would be elucidated by nuclear magnetic resonance spectroscopy and mass spectroscopy.

CHAPTER 2

MATERIALs & METHODs

2.1. Materials

2.1.1. Materials for Synthesis

TEMPO (2,2,6,6-tetramethyl-1-piperidinuyloxy, Acr), NaOCl (Sodium hypocholoride, CE), NaBr (Sodium Bromide, CE), DIPEA (Diisopropylethylamine, Merck MW: 742 g/mol), HOBt (Hydroxylbenzotroazole, Aldrich MW: 153.134 g/mol), EDC [(1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, TCI, MW: 191.70 g/mol] and Dodecylamine (Merck- MW: 185.355 g/mol), Pyridine (Sigma) were purchased for semi-synthesis studies. AST VII was donated by Bionorm Doğal Ürünler, Izmir, TURKEY. Chloroform (VWR Chemicals), methanol (Carlo Erba), ethyl acetate (VWR Chemicals) and acetonitrile (VWR Chemicals) were purchased for chromatography studies.

2.1.2. Materials for Bioactivity Studies

RPMI 1640 medium (Gibco, USA), ISCOVE's Complete Medium (Thermo), Fetal Bovine Serum (FBS) (Biochrom), Dulbecco's Phosphate Buffer Saline (DPBS) (Gibco), 2-Mercaptoethanol (Aldrich), Penicillin/Streptomycin (Gibco), GM-CSF (Granulocyte Macrophage Colony Stimulating Factor) (R-D), M-CSF (Macrophage Colony Stimulating Factor) (Peprotech), Lipopolysaccharide (LPS) (invivogen) Mouse CD16/32 (Fc Block) (Tonbo Biosciences), APC conjugated anti-mouse CD11c (Biolegend, PE-Cy7 conjugated anti-mouse MHC (I-A/I-E)(eBioscience), Pacific Blue conjugated anti-mouse CD80 (Biolegend), PerCp Cy5.5 conjugated anti-mouse CD86 (Biolegend), PE conjugated anti-mouse F4/80, PE-Cy7 conjugated CD86 (Biolegend), APC-Fire750 conjugated anti-mouse MHC (I-A/I-E) (Biolegend), Fixation Buffer (BD), Mouse IL-12p70 ELISA kits (eBioscience), Mouse IL-2 ELISA kits (eBioscience),

Human IL-2, IL-4, IL-17A, TNF-α, IFN-γ, IL-1β ELISA kits (eBioscience), CPRG (Chlorophenol Red-β-D-galactropyranoside) (Sigma), NP40S (Sigma), SIINFEKL (OVA 257-264) (Sigma), OVA soluble protein (invivogen), EasySep Mouse Naive $CD4^+$ T cell and $CD8^+$ T cell Isolation Kit (StemCell), Bovine Serum Albumin (BSA) (PanReac Applichem), Sodium azide (Sigma), ELISA Wash Buffer (Biolegend) and Stop Solution for TMB (Biolegend) were purchased for bioactivity studies. QS-21 was purchased from Desert King International (San Diego, CA, USA).

2.1.3. Buffers

a) FACS (Fluorescence Activated Cell Sorting) Buffer: 5 g BSA (Bovine Serum Albumin) and 125 mg sodium azide were dissolved in 500 mL 1X DPBS, and continuously mixed to be used in flow cytometry analysis.

b) Z Buffer 10 mL of Z buffer was prepared by dissolving 90 µL $MgCl_2$ (1 M), 125 µL 10% NP-40, 70 µL 2-ME (14.3 M) and 10 µL CPRG (150 mM) in 9.7 mL 1X DPBS.

c) Wash Buffer (1X): Commercial wash buffer (5X) was diluted with DPBS to give 1X wash buffer.

2.1.4. Cells

Mouse bone marrow derived dendritic cells (BMDCs) were maintained in R5 medium containing RPMI 1640 medium with 5% Fetal Bovine Serum, 50 U/mL Penicillin/streptomycin with 5 ng/mL GM-CSF. Mouse bone marrow derived macrophages (BMDMs) were cultured in R5 medium: L929 medium (1:1) supplemented with 10 ng/mL M-CSF. B3Z, co-stimulation independent CD8+ T cell hybridoma cell line, specific for the SIINFEKL (OVA257–264) peptide of OVA111 was cultured in ISCOVE's medium supplemented with 8% FBS, 1% Pen/Strep., 50 µM 2-mercaptoethanol. B3Z cells splitted 2-3 times in a week. Naive CD4+ and CD8+ T cells were isolated from B6 mice with negative isolation kits, and maintained in R5 medium.

2.1.5. Experimental Animals

C57BL/6 and Balb/c mice were provided by Izmir Biomedicine and Genome Center (IBG) and used for isolation of bone marrow and spleen. All mice were kept in a specific pathogen-free animal facility at IBG and maintained in groups of 5 under standard conditions of temperature 22 ± 1°C with regular 12 h light and 12 h dark cycles and had free access to standard laboratory food and water. The experimental protocol was approved by the Local Ethics Review Committee for Animal Experimentation of IBG.

2.1.6. Instruments

Nuclear Magnetic Resonance Spectrometer Varian MERCURY*plus*-AS 400 (400 MHz)
Agilent 1200/6530 Instrument-HRTOFMS
Lyophilizer (Christ, Alpha1-2LDplus)
Rotavapor (Heidolph)
Flow Cytometry (BD CantoII)
96-well plate reader (ThermoFisher)
UV Lamp (Vilber Lourmat)

2.2. Methods

2.2.1. Semi-Synthesis Studies Performed on AST VII

2.2.1.1. Oxidation of Primary Alcohols to Carboxylic Acids

Anneli's oxidation protocol was followed to oxidize primary alcohols to carboxylic acids.[112] AST VII (1000 mg, 1.06 mmol, 1 equiv.) and NaBr (109 mg, 1.06 mmol, 1 equiv.) were dissolved in distilled water (pH 11 adjusted with 1 N NaOH) and mixed. TEMPO (40 mg, 0.212 mmol, 0.2 equiv.) was added to the reaction mixture. NaOCl (6.3 mL, 4.66 mmol, 4.4 equiv) was slowly added to the reaction mixture

dropwise and stirred at 0°C. After 6 hours, the reaction mixture was quenched by addition of distilled water. Water fraction was neutralized by 1 M HCl, extracted with *n*-butanol and evaporated at 50°C in rotary evaporator for further separation and purification steps.

2.2.1.2. Conjugation of Free Dodecylamine to the Dicarboxylic Analog of AST VII

Free amino group of dodecylamine was conjugated to the glucuronic acid carboxyl of AST-VII via amide formation. Dicarboxylic AST-VII (50 mg, 0.0513 mmol, 1 equivalent) was dissolved in pyridine. DIPEA (27 mg, 0.2052 mmol, 4 equiv.), HOBt (16 mg, 0.1026 mmol, 2 equiv.), EDC (30 mg, 0.1539 mmol, 3 equiv.) were added and the reaction mixture was stirred for 1 hour at room temperature. One hour later, free dodecylamine (23 mg, 0.1231 mmol, 2.4 equiv.) was added and mixed at room temperature for overnight. Next day, the temperature was increased to 60°C. All reagents were added to the reaction mixture in excess amount and reaction was continued until dicarboxylic AST-VII was consumed. The reaction quenched by addition of distilled water and was extracted with ethyl acetate. Ethyl acetate fraction was evaporated at 50°C in rotary evaporator for further separation and purification steps.

2.2.2. Separation and Purification of Semi-Synthetic Analogs

Extraction of the semi-synthetic products from the reaction mixture was performed with ethyl acetate (EtOAc) and concentrated by rotary evaporator at 50 °C. Column chromatography was carried out on Silica gel (Kiesegel 60, 70-230 mesh, Sigma), Silica gel RP-18 (LiChroprep C18), Sephadex LH-20 (GE-Healthcare). Silica gel (Aluminium sheets, Kiesegel 60 F_{254}, 0.2 mm, Merck) and RP silica gel (C-18) (Aluminium sheets, 0.2 mm, Kiesegel 60 RP-18 F_{254}, Merck) coated commercial plates were used for evaluation of extraction and chromatographic fractionation/purification stages.

TLC bands were marked under the UV light at 254 and 366 nm, and then sprayed with 20% aq. H_2SO_4 and heated for 5 minutes to visualize the bands. Dragendorff's reagent, which was prepared in distilled water (100 mL) by mixing bismuth carbonate (Bi_2CO_3, 5 g), concentrated HCl (10 mL) and potassium iodide (KI - 25 g) was used to visualize the synthesized amides.

During chromatographic studies the following solvent systems were used;

I	$CHCl_3$:MeOH:H_2O	80:20:2
II	EtOAc:MeOH:H_2O	100:25:15
III	MeOH:H_2O	15:85
IV	MeOH: H_2O	20:80
V	MeOH:H_2O	35:65
VI	MeOH:H_2O	40:60
VII	MeOH:H_2O	50:50
VIII	MeOH:H_2O	55:40
IV	ACN:H_2O	5:95
X	ACN:H_2O	10:90

2.2.2.1. Separation and Purification of Dicarboxylic AST VII (DC-AST VII)

The reaction quenched at 2 hours and 4 hours by addition of distilled water, neutralized by 1 M HCl and evaporated at 50°C in rotary evaporator. Silica gel TLC chromatogram of the TEMPO mediated oxidation reaction was shown in Figure 2.1. According to this chromatogram, isolation studies of DC-AST VII were performed on reversed phase (C-18) vacuum liquid chromatography and silica gel chromatography.

Reaction mixture at 2 hours was subjected to vacuum liquid chromatography (VLC) using reversed-phase material (Lichroprep RP-18, 25 g) employing H_2O (250 mL), MeOH: H_2O (15:85, 100 mL; 20:80, 100 mL; 35:65, 100 mL; 40:60, 100 mL; 50:50, 100 mL; 55:45, 100 mL) and MeOH (250 mL) to give 113 fractions. Subfraction 53-71 was rich in DC-AST VII (240 mg).

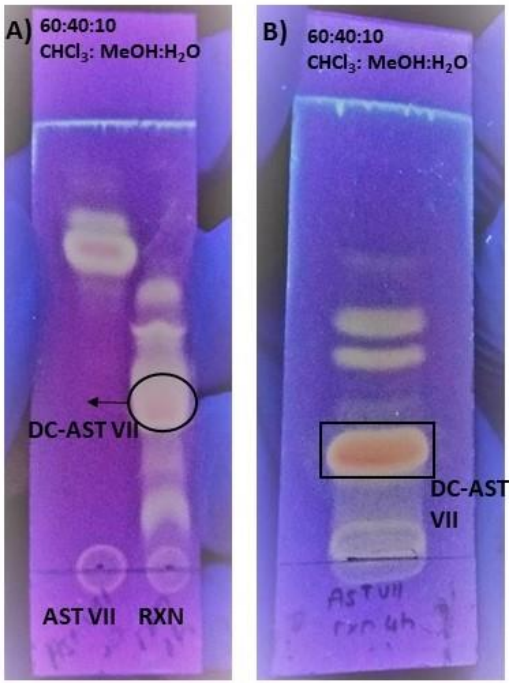


Figure 2.1. Silica gel TLC chromatogram of the TEMPO mediated oxidation reaction of AST VII [Mobile Phase: $CHCl_3$:MeOH:H_2O (60:40:10)]. 2.1.A illustrated TEMPO reaction quenched at 2 hours. 2.1.B illustrated TEMPO reaction quenched at 4 hours.

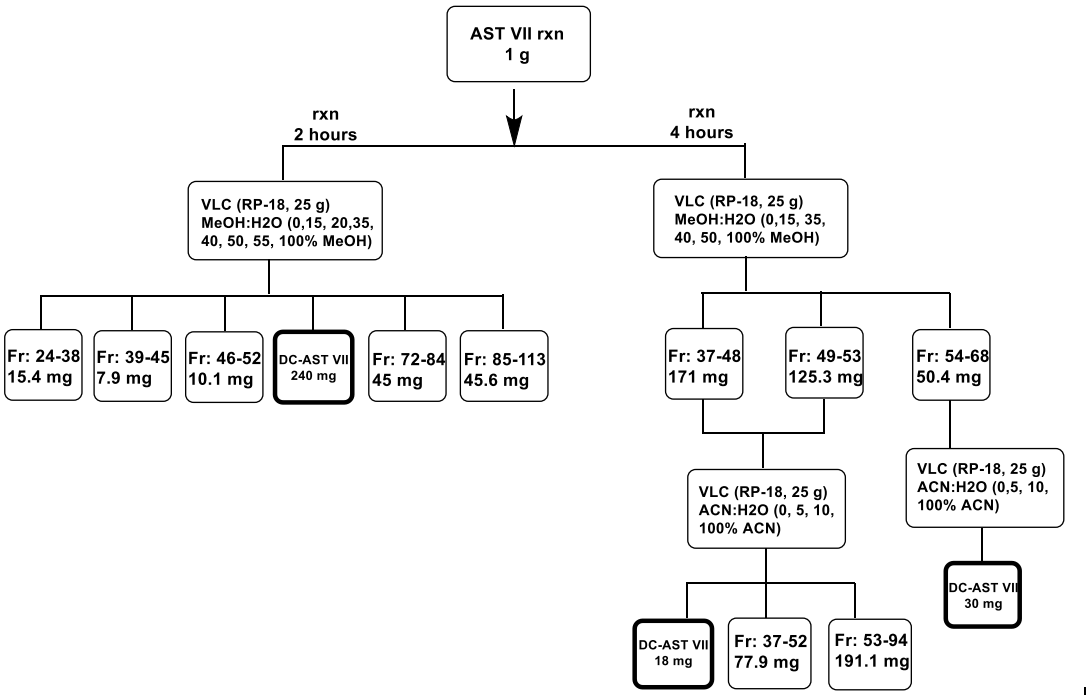


Figure 2.2. Isolation Procedure of DC-AST VII

Reaction mixture at 4 hours was subjected to VLC (Lichroprep RP-18, 25 g) employing H_2O (250 mL), MeOH:H_2O (15:85, 100 mL; 35:65, 100 mL; 40:60, 100 mL; 50:50, 100 mL) and MeOH (250 mL) to give 68 fractions. Fractions 37-48 and 49-53 combined and applied to VLC using reversed-phase material (Lichroprep RP-18, 25 g) employing H_2O (250 mL), ACN:H_2O (5:95, 300 mL; 10:90, 200 mL) and ACN (250 mL) to give DC-AST VII (18 mg) and 94 fractions.

Fraction 54-68 (50.4 mg) was also applied to reversed-phase VLC (Lichroprep RP-18, 25 g) employing H_2O (250 mL), ACN:H_2O (5:95, 200 mL; 10:90, 100 mL) and ACN (250 mL) to give DC-AST VII (30 mg). Isolation procedure of DC-AST VII was illustrated in Figure 2.2.

2.2.2.2. Separation and Purification of Dodecylamine Conjugated AST VII (DAC-AST VII)

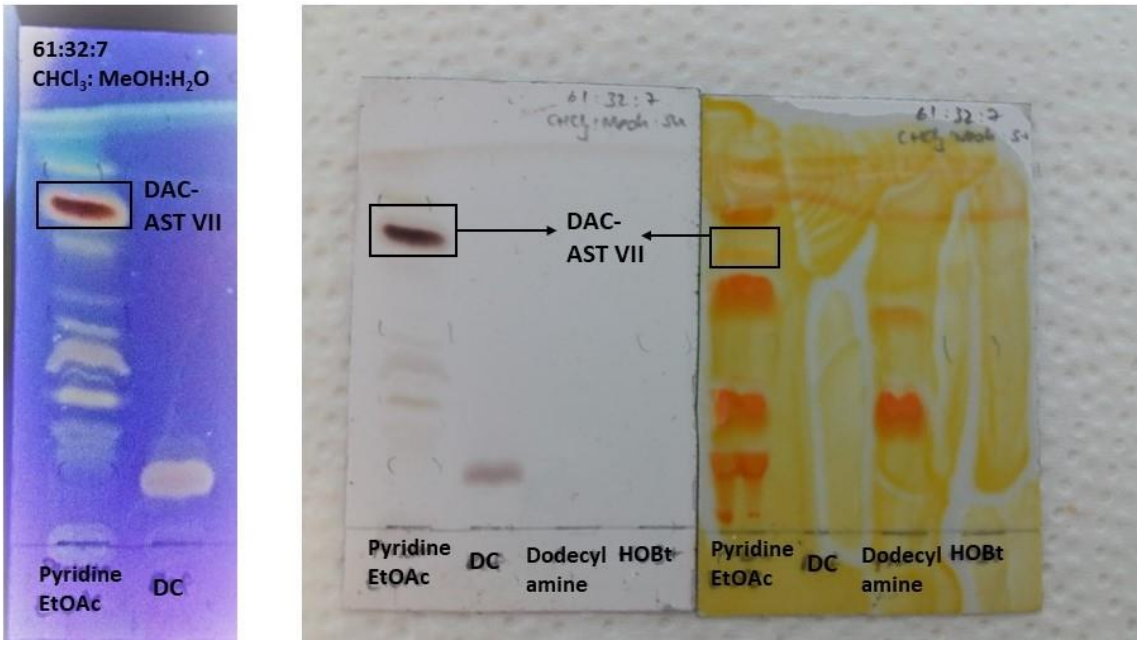


Figure 2.3. Silica gel TLC chromatogram of the DC-AST VII rxn [Mobile Phase: $CHCl_3$:MeOH:H_2O (60:40:10)]

The reaction quenched by addition of distilled water and extracted 3 times with ethyl acetate. The combined ethyl acetate fraction was evaporated at 50°C in a rotary evaporator. Silica gel TLC chromatogram of the DC-AST VII reaction was shown in

Figure 2.3. Based on these chromatograms, the isolation studies of DAC-AST VII from the EtOAc extract were carried out by means of successive Sephadex LH-20 and silica gel chromatographies. EtOAc extract (88.7 mg) was given to Sephadex LH-20 column and eluted with MeOH to give 15 fractions. Fractions 5-6 (28.1 mg) was applied to silica gel column chromatography (25 g) and eluted with EtOAc:MeOH:H_2O (100:25:15). Sub-fraction 3 (13.4 mg) was subjected to silica gel column chromatography (15 g) employing $CHCl_3$:MeOH:H_2O (80:20:2) to isolate DAC-AST VII (5.1 mg).

Fraction 7-15 (37.7 mg) was fractionated by silica gel column chromatography (30 g) and eluted with $CHCl_3$:MeOH:H_2O (80:20:2) to give DAC-AST VII (8.8 mg). Isolation procedure of DAC-AST VII was shown in Figure 2.4.

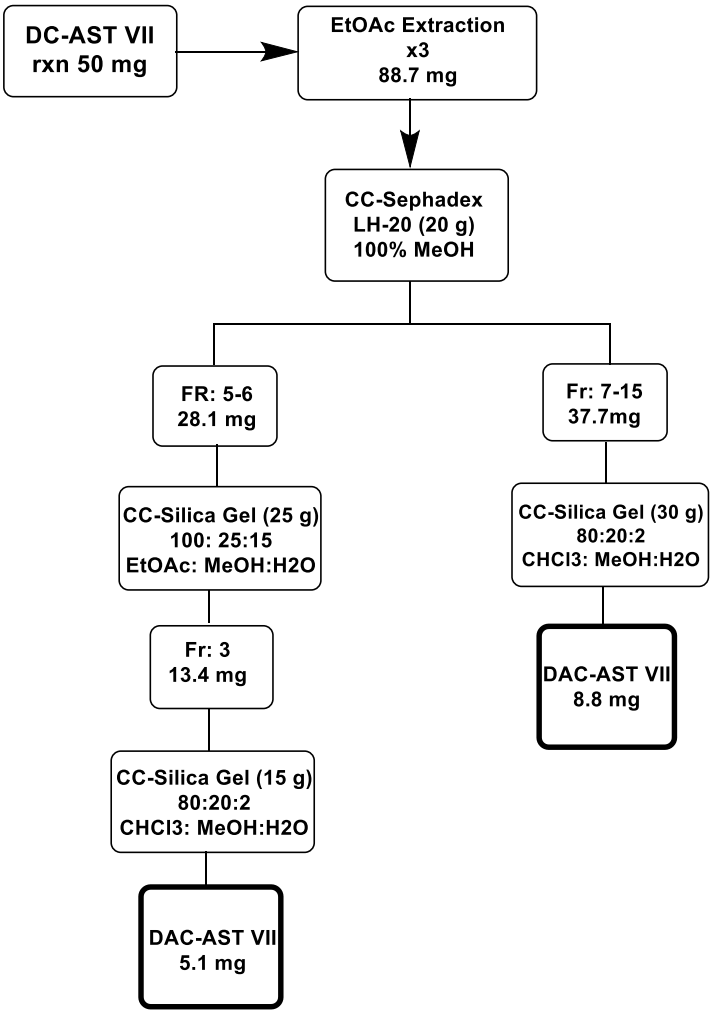


Figure 2.4. Isolation Procedure of DAC-AST VII

2.2.3. Whole Blood Stimulation Assay

Whole blood stimulation assay was performed to elucidate the cytokine release profiles of AST VII analogs (DC-AST VII and DAC-AST VII) in parallel to AST-VII and QS-21. Heparinized whole blood from healthy volunteers was supplemented in 1:10 and 1:20 with RPMI-1640 medium, 100 U/mL penicillin/streptomycin and 10% fetal bovine serum. PMA (50 ng/mL) and ionomycin (400 ng/mL) were also added to the whole blood suspensions. One mL of this solution was transferred into each well of a 24-well plate. AST-VII, DC-AST VII, DAC-AST VII and QS-21 at the concentrations of 2, 4, 8, 16, 32 μg/mL were administrated after stimulation with PMA/ionomycin. Finally, the plate was incubated at 37°C in 5% CO_2 for 48 h. Supernatants of each well were collected and stored at -20°C for ELISA.

2.2.4. Generation of Bone Marrow Derived Dendritic Cells (BMDCs) and Bone Marrow Derived Macrophages (BMDMs)

Femurs and tibias of C57BL/6 and Balb/c mice were collected and flushed with a sterile DPBS twice. The resulting bone morrow cells were resuspended RPMI 1640 medium containing 5% heat-inactivated FBS, 2 mM L-glutamine, 50 U/mL penicillin/streptomycin plus 5 ng/mL GM-CSF. A total of $2x10^6$ cells per bacteriological culture plate were cultured for 10 days by feeding on days 3 and 8 with fresh medium, and on day 6 by centrifugal feeding to new plate. Nonadherent and loosely adherent cells were collected on day 10 and resulting cell population was 79% $CD11c^+$.

For the generation of BMDMs, same protocol of BMDCs was followed. After isolation of bone marrow cells, they cultured for 1 day in the tissue culture plate supplemented with R5:L929 (1:1) medium with 10 ng/mL M-CSF. Next day, the cells were collected and transferred to 6 well low attachment plate. On day 6, the media was refreshed and on day 8 the cell were collected and centrifuged at 300 rcf (relative centrifugation force) for 5 minutes. The pellet was suspended with R5 medium and slowly transferred over the Ficoll solution in the dark. The falcon tube was centrifuged at 300 rcf for 20 minutes with no brake. BMDMs between Ficoll and R5 medium was taken at one move.

2.2.5. Stimulation of BMDCs and BMDMs with Adjuvants

BMDCs were suspended in R5 medium and cultured in 96 well plate at concentration of $2.5x10^5$ BMDC/well in 200 μL one day before the experiment. AST VII was dissolved in DPBS and filtered through 0.22 μm filter. Different concentrations of AST VII (1; 3; 6; 11; 16; 21; 32; 64 μM) were added to the appropriate wells and incubated 24 hours at 37°C in 5% CO_2 incubator.

BMDCs were treated with LPS (10 ng/mL) and different concentrations of AST VII, DC-AST VII and DAC-AST VII (2; 5; 10 μM) for upregulation of MHC II, CD86, CD80 and incubated 24 hours at 37°C in 5% CO_2 incubator to reveal the additive effect of LPS and the adjuvants. Moreover, DC-AST VII and DAC-AST VII (2; 5; 10 μM) were administrated to BMDCs without LPS stimulation. All samples were studied in triplicate. Similar experimental set was made by treating cells with 0.5; 2.5; 10 μM AST VII, DC-AST VII and DAC-AST VII following LPS stimulation. The supernatant was collected in 6 hours to measure IL-1β cytokine release.

BMDMs were plated as $1x10^5$ BMDMs/well in 200 μL one day before the experiment. The cells were treated with LPS (10 ng/mL) and different concentrations of AST VII, DC-AST VII and DAC-AST VII (2; 5; 10 μM) for IL-1β cytokine release. All samples were studied in triplicate.

2.2.6. Stimulation of Splenocyte Suspension with Adjuvants

Spleens from C57BL/6 mice were collected and single cell suspensions were prepared by gently mincing and grinding the spleen fragment in R5 medium on a fine steel mesh under aseptic conditions. Splenocyte were cultured in 96 well plate at concentration of $3x10^5$ BMDC/well in 200 μL. AST VII (5; 11; 21 μM) was stimulated as 1% of the total volume and incubated 20 hours at 37°C in 5% CO_2 incubator. Splenocytes were treated with LPS (1 ng/mL) and different concentrations of AST VII (5; 11; 21 μM) and then incubated 24 hours at 37°C in 5% CO_2 incubator to reveal the additive effect of LPS and the adjuvants. LPS (10 ng/mL) was used as a positive control. All samples were studied in triplicate.

2.2.7. Evaluation of Co-Stimulation Independent Cross-Presentation by B3Z Assay

$5x10^4$ BMDCs/well were plated to 96 well microplate 24 hours before the experiment. The plate was centrifuged at 300 rcf for 5 minutes at room temperature. Supernatant was discarded, and stimulants were added to the appropriate wells. Ovalbumin (OVA) at concentrations of 10, 30, 60 and 90 μg/mL and SIINFEKL at 10 ng/mL were administrated in the presence/absence of AST VII. Antigen and adjuvant induced BMDCs were incubated for 5 hours at 37°C in 5% CO_2 incubator. After 5 hours, BMDCs were washed with DPBS twice to remove excess amounts of OVA. B3Z cells were cultured in 75 cm^2 T flask with complete ISCOVE's media at least 4 days before the experiment. $5x10^4$ B3Z/well was added as 1:1 effector:target cell ratio and incubated for 18 hours at 37°C in 5% CO_2 incubator. B3Z readout was analyzed with bulk LacZ assay. After 18 hours, the plate was centrifuged at 600 rcf for 3 minutes. Supernatant was kept at -20°C for subsequent cytokine analysis. The plate was washed with DPBS, centrifuged and flicked. 100 μL Z buffer was added and incubated at 37°C. The plate was checked every 30 minutes until the solution changed its color to orange/red. The OD value of plate was measured at 595 nm every 30-60 minutes until the reaction saturated (after 2-4 hours incubation).

2.2.8. Naive $CD4^+$ and $CD8^+$ T Cell Isolation

C57BL/6 mice were euthanized, and spleens were collected and disrupted in DPBS containing 2% FBS. Single splenocyte suspensions were obtained by passing through a 70 μm mesh nylon strainer. The cell suspension was centrifuged at 300 rcf for 10 minutes three times and resuspended at $1x10^8$ nucleated cells/mL in DDPBS containing 2% FBS. Naive $CD4^+$ and $CD8^+$ T cells were obtained by using negative selection StemCell EasySep™ Mouse Naive $CD8^+$ and $CD4^+$ T Cell Isolation Kit as suggested by the manufacturer.

According to kit protocol, $1x10^8$ splenocyte in 1 mL was placed to a 5 mL (12 x 75 mm) polystyrene round bottom tube. 50 μL $CD8^+$ Isolation cocktail was added to the sample, mixed and incubated at RT for 10 minutes. RapidSpheres™ was vortexed until particles appeared evenly dispersed and added in 100 μL to the sample, mixed and

incubated 5 minutes. DPBS containing 2% FBS added to top up the sample to 2.5 mL and gently mixed by pipetting up and down 2-3 times. The tube was placed into a magnet and incubated at RT for 2.5 minutes. The magnet was picked up, the magnet and the tube were inverted in one continuous motion and the enriched naive $CD8^+$ T cell suspension was poured into a new tube.

Mouse Naive $CD4^+$ T cells were isolated with similar protocol as $CD8^+$ T cell proliferation. $1x10^8$ splenocyte in 1 mL was placed to a 5 mL (12 x 75 mm) polystyrene round bottom tube. 50 μL $CD4^+$ Isolation cocktail was added to the sample, mixed and incubated at RT for 7.5 minutes. 50 μL/mL depletion cocktail was added to the sample, mixed and incubated for 2.5 minutes. RapidSpheres™ was vortexed until particles appeared evenly dispersed and added in 75 μL to the sample, mixed and incubated for 2.5 minutes. DPBS containing 2% FBS added to top up the sample to 2.5 mL and gently mixed by pipetting up and down 2-3 times. The tube was placed into a magnet and incubated at RT for 2.5 minutes. The magnet was picked up, the magnet and the tube were inverted in one continuous motion and the enriched naive $CD4^+$ T cell suspension was poured into a new tube.

2.2.9. Evaluation of T Cell Presentation by Mix Lymphocyte Reaction (MLR)

Allogenic-MLR protocol was used to analyze the T cell proliferating and polarizing potential of DCs. BMDCs were generated from Balb/c mice with the described protocol. Harvested BMDCs were plated $2x10^4$ cells/well to 96 well plate in 100 μL R5 medium. LPS (10 ng/mL), AST VII (5 μM), DC (5 and 10 μM), DA (5 and 10 μM) were added and incubated for 24 hours at 37°C in 5% CO_2 incubator. Next day, the plate was washed with DPBS for removing excess amounts of stimulants. $1x10^5$ isolated naive $CD4^+$ and $CD8^+$ T cells/well were added to the 96 well plate in 200 μL. The plate was incubated for 3 days at 37°C in 5% CO_2 incubator. The supernatant was kept at -20°C for subsequent cytokine analysis. The cells were stained for analysis in flow cytometry. $CD4^+$ and $CD8^+$ T cell activation were assessed as expression of CD44. Data were presented as mean fluorescence intensity (MFI) for CD44.

2.2.10. Cell Surface Marker Staining for Flow Cytometry Analysis

The cells were centrifuged at 300 rcf for 5 minutes at 4°C. The supernatants were collected and stored at -20°C for ELISA. The cells were detached harsh up and down with acutase and washed twice with FACS Buffer. The plate was flicked after centrifugation and 1/200 diluted mouse CD16/32 (Fc block) in 50 µL FACS buffer added to each well and incubated for 10 minutes on ice. Cell surface staining dye cocktail was prepared in FACS buffer and added directly to 50 µL Fc block as final dye concentration was 1/400. The plate was incubated at dark on ice for 40 minutes. For compensation, 0.5 µL from each dye was added as final dye concentration was 1/400. The plate was washed twice with FACS buffer and centrifuged 300 rcf for 5 minutes at 4°C to stop staining. 100 µL cytofix-fixation medium was added and incubated for 15 minutes. After that, the cells were washed and resuspended in FACS buffer in 200 µL. Flow cytometry was performed using BD Aria III followed by analysis in FlowJo™.

2.2.11. ELISA

Cytokine concentrations of human IL-2, IFN-γ, IL-4, IL-17A, TNF-α, IL-1β and mouse IL-2, IL-12 were determined by using commercial ELISA kits (e-Bioscience, Austria). All steps were performed in accordance with the manufacturer's instructions. The plate was coated with appropriate capture antibody in coating buffer and incubated overnight at 4°C. The plate was washed with washing buffer 4 times and blocked by adding 200 µL 1X assay diluent to each well, sealed and incubated at room temperature for 1 hour with shaking on a plate shaker. After washing step, 100 µL diluted samples and standards were added to appropriate wells and incubated for 2 hours with shaking. 100 µL diluted detection antibody solution was transferred and incubated for 30 minutes. 100 µL of freshly mixed TMB substrate solution was added and incubated in the dark for 30 minutes. 100 µL stop solution was added to each well and read absorbance at 450 nm and 570 nm within 15 minutes. The absorbance at 570 nm was subtracted from the absorbance at 450 nm. The plate was washed at least 4 times in each step.

2.2.12. Statistics

The data was measured with mean standard errors and the statistical significance of differences was examined by using Student-t test, One-way ANOVA (GraphPad Prism 5.01). P values of less than 0.05*, 0.01** and 0.001*** were stated as statistically significant.

CHAPTER 3

RESULTS & DISCUSSION

3.1. Structural Identification of DC-AST VII

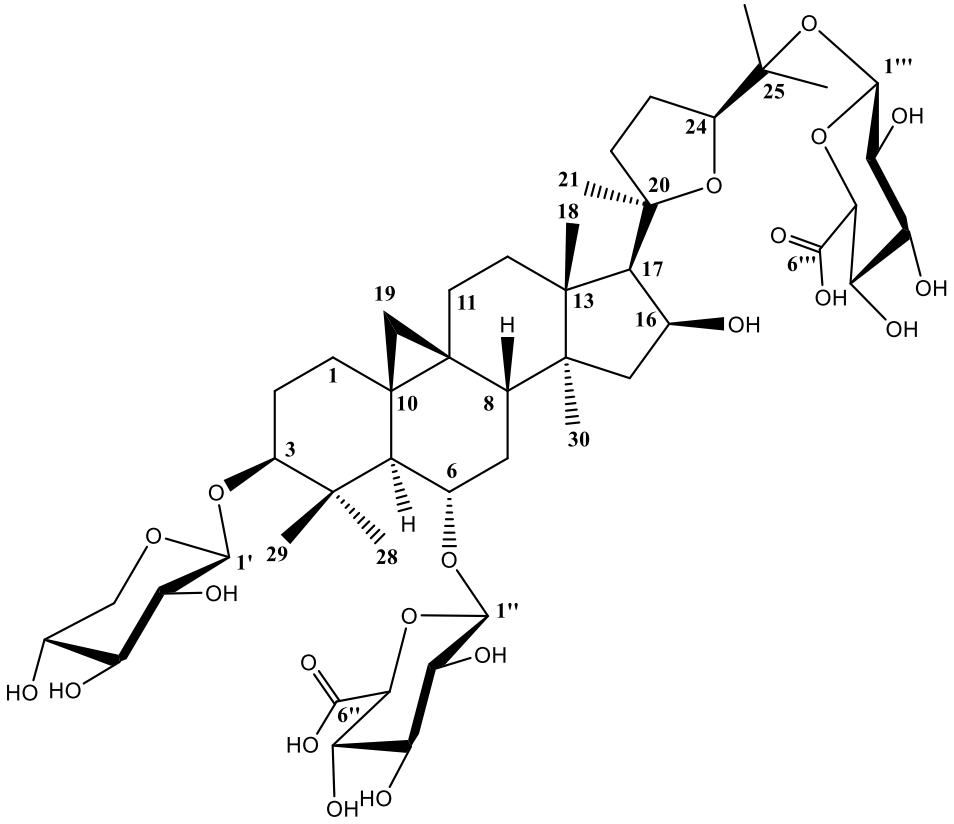


Figure 3.1. Chemical Structure of Dicarboxylic AST VII (DC-AST VII)

IUPAC name:

(2S,3S,4S,5R,6S)-6-((2-((2S,5R)-5-((2aR,3R,4S,5aS,5bS,7S,7aR,9S,11aR,12aS)-7-(((2R,3R,4S,5S,6S)-6-carboxy-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)oxy)-4-hydroxy-2a,5a,8,8-tetramethyl-9-(((2S,3R,4S,5R)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)oxy)tetradecahydro-1H,12H-cyclopenta[a]cyclopropa[e]phenanthren-3-yl)-5-methyltetrahydrofuran-2-yl)propan-2-yl)oxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxylic acid

Molecular Formula: $C_{47}H_{74}O_{21}$

Exact Mass: 974.47

A major ion peak was observed at m/z 995.38897 $[M+Na-2H]^-$ indicating the molecular formula as $C_{47}H_{74}O_{21}$ (calculated 995.444 for $C_{47}H_{72}O_{21}Na$) in the HR-ESI-MS spectrum (negative mode) of DC-AST VII. A detailed comparison of NMR data (^1H, ^{13}C, COSY, HSQC and HMBC) of DC-AST VII demonstrated that its aglycone was similar with AST VII. The ^1H NMR spectrum showed signals due to a cyclopropane methylene at δ 0.70 and 0.40 (each 1H, d, *J*= 3.8, 4.2 Hz), seven tertiary methyl groups at 1.39 (3H, s), 1.32 (3H, s), 1.32 (3H, s), 1.30 (3H, s), 1.29 (3H, s), 1.06 (3H, s), 1.01 (3H, s). The resonation of three anomeric protons and carbons [δ 4.50, d, *J*=7.8 Hz, 1H; δ 4.56, d, *J*=7.9 Hz, 1H; δ 4.70, d, *J*=8.1 Hz, 1H; δ 105.5 d, δ 102.5 d, 96.7 d, respectively] of DC-AST VII were in good agreement with AST VII.[113] Glycosidation shifts for the aglycone moiety were observed for C-3 (δ 3.39 m, 89.3), C-6 (δ 3.69 m, 80.3) and C-25 (δ 79.7). Key correlation peaks in HMBC spectrum were determined between the proton signals at δ 4.50 (H-1') and the carbon resonance at δ 89.3 (C-3), at δ 4.56 (H-1'') and δ 80.3 (C-6), at δ 4.70 (H-1''') and δ 79.7 (C-25). The major difference was the downshift of the C-6'' [from δ 62.5 to δ 176.1] and C-6''' [from δ 62.5 to δ 175.7], indicating the oxidation of the primary alcohols at C-6'' and C-6''' to carboxylic acids. In HMBC spectrum, strong three bond correlation between the carbon resonance at 176.1 (C-6'') and the proton signal at δ 3.72 (H-4''), δ 3.75 (H-5''); weak two-bond correlations between 175.7 (C-6''') and the proton signal at δ 3.72 (H-4'''), δ 3.69 (H-5''') was proved the oxidation at C-6 on the glucose moities. The structure of DC-AST VII was established as 3-*O*-β-D-xylopyronosyl-6,25-di-*O*-β-D-glucuronopyronosyl-20(*R*),24(*S*)-epoksi-3β-6α-16β-25-tetrahydroxycycloartane.

Table 3.1. The ^{13}C and ^1H NMR data of DC-AST VII (100/400 MHz, δ ppm, in D_2O)

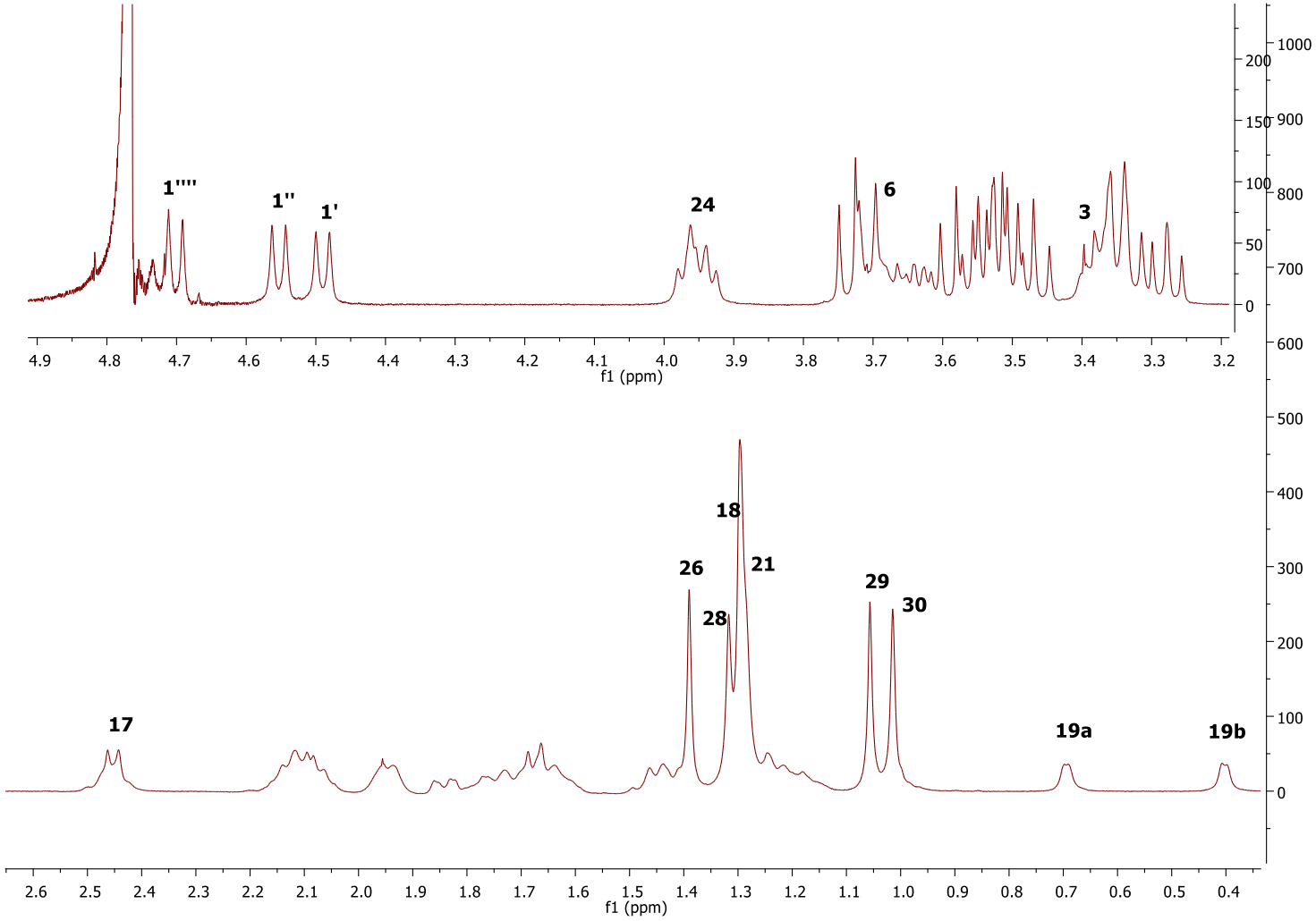
H/C	δ_C (ppm)	δ_H (ppm), *J* (Hz)
1	31.9 t	1.22 m, 1.67 m
2	29.2 t	1.68 m, 1.96 m
3	89.3 d	3.39 m
4	41.5 s	
5	51.6 d	1.68 m
6	80.3 d	3.69 m

(cont. on next page)

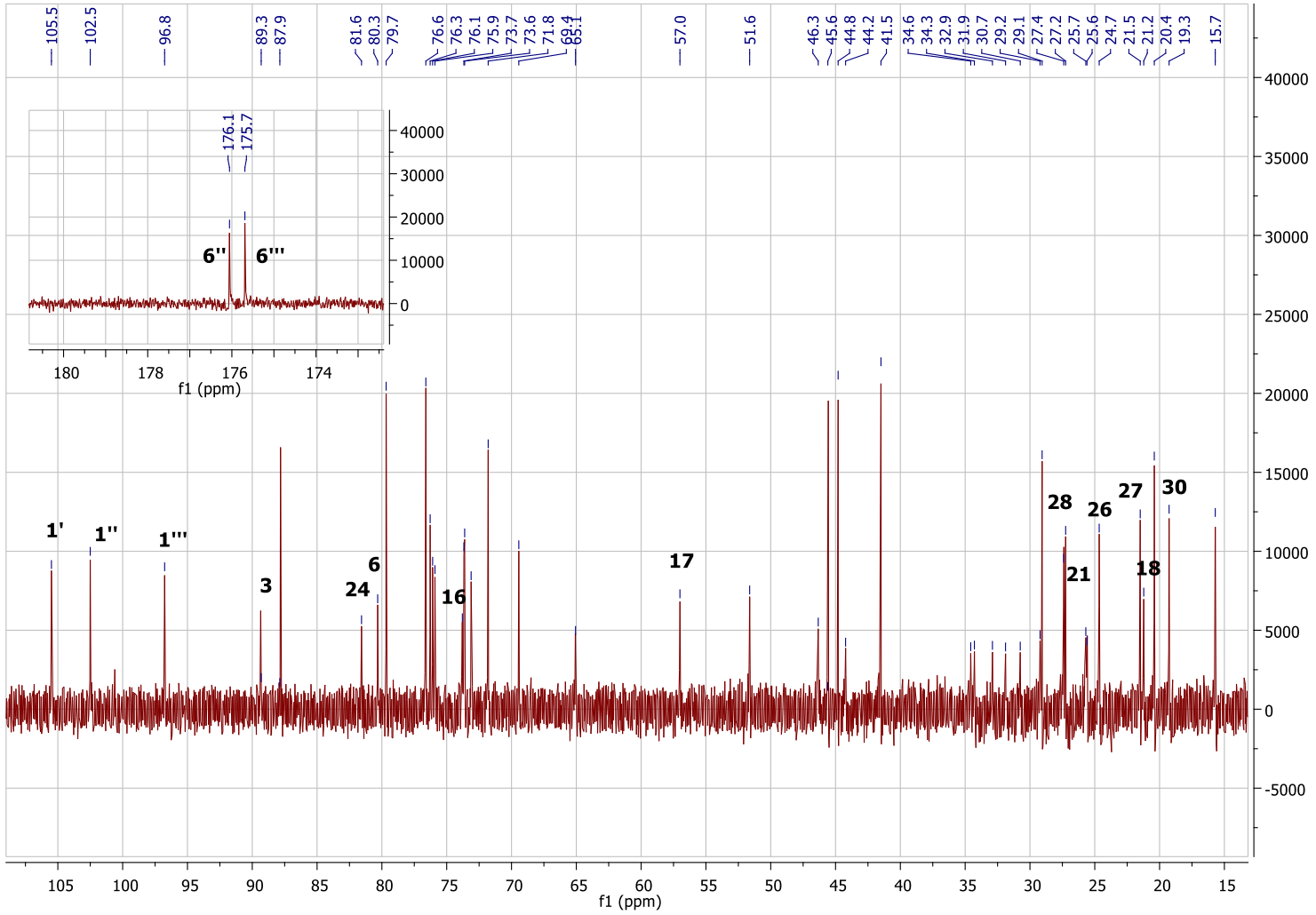
Table 3.1. (cont.)

H/C	δ_C (ppm)	δ_H (ppm), *J* (Hz)
7	34.6 t	1.45 m, 1.94 m
8	46.3 d	1.84 m
9	20.4 s	
10	29.1 s	
11	25.7	1.17 m, 1.94 m
12	32.9 t	1.62 m, 1.74 m
13	45.6 s	
14	44.8 s	
15	44.2 t	1.44 m, 2.08 m
16	73.8 d	4.72 dd (6, 4.5)
17	57.0 d	2.46 d (8)
18	21.2 q	1.29 s
19	30.7 t	0.40 s, 0.70 s
20	87.85 s	
21	27.3 q	1.29 s
22	34.3 t	1.75 m, 2.46 m
23	25.6 t	2.12 m, 2.16 m
24	81.6 d	3.97 d
25	79.7 s	
26	24.7 q	1.39 s
27	21.5 q	1.30 s
28	27.4 q	1.32 s
29	15.7 q	1.06 s
30	19.3 q	1.01 s
1'	105.5 d	4.50 d (7.8)
2'	73.6 d*	3.32 m
3'	76.1 d^+	3.46 m
4'	69.4 d	3.64 m
5'	65.1 t	3.33 d, 3.94 d
1''	102.5 d	4.56 d (7.9)
2''	73.1 d	3.33 m
3''	75.9 d^+	3.50 m
4''	71.8 d	3.72 m
5''	76.6 d	3.75 m
6''	176.1 s	
1'''	96.8 d	4.70 d (8.1)
2'''	73.7 d*	3.26 m
3'''	75.9 d^+	3.46 m
4'''	71.8 d	3.69 m
5'''	76.3 d	3.72 m
6'''	175.7 s	

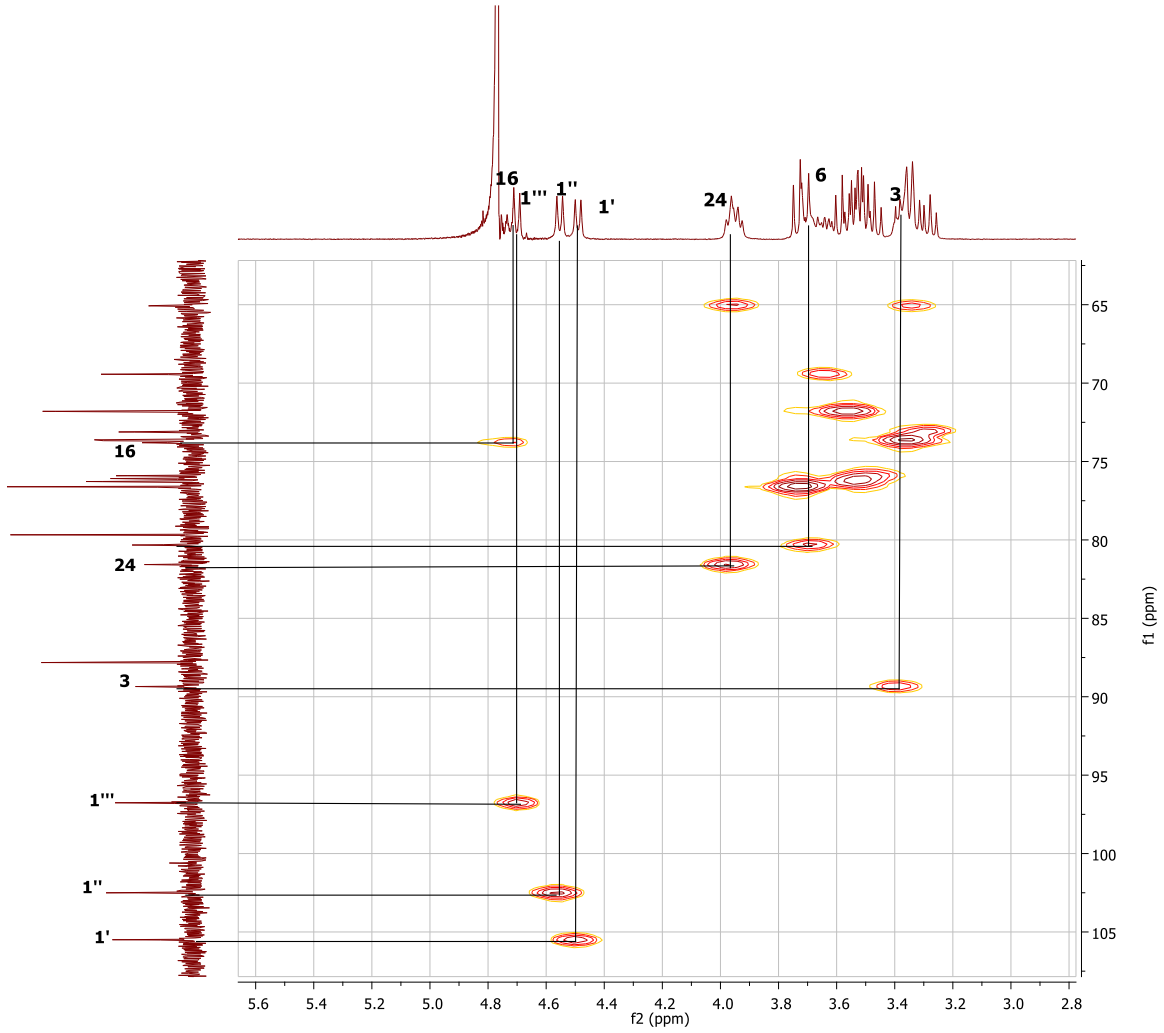
*,$^+$: changeables



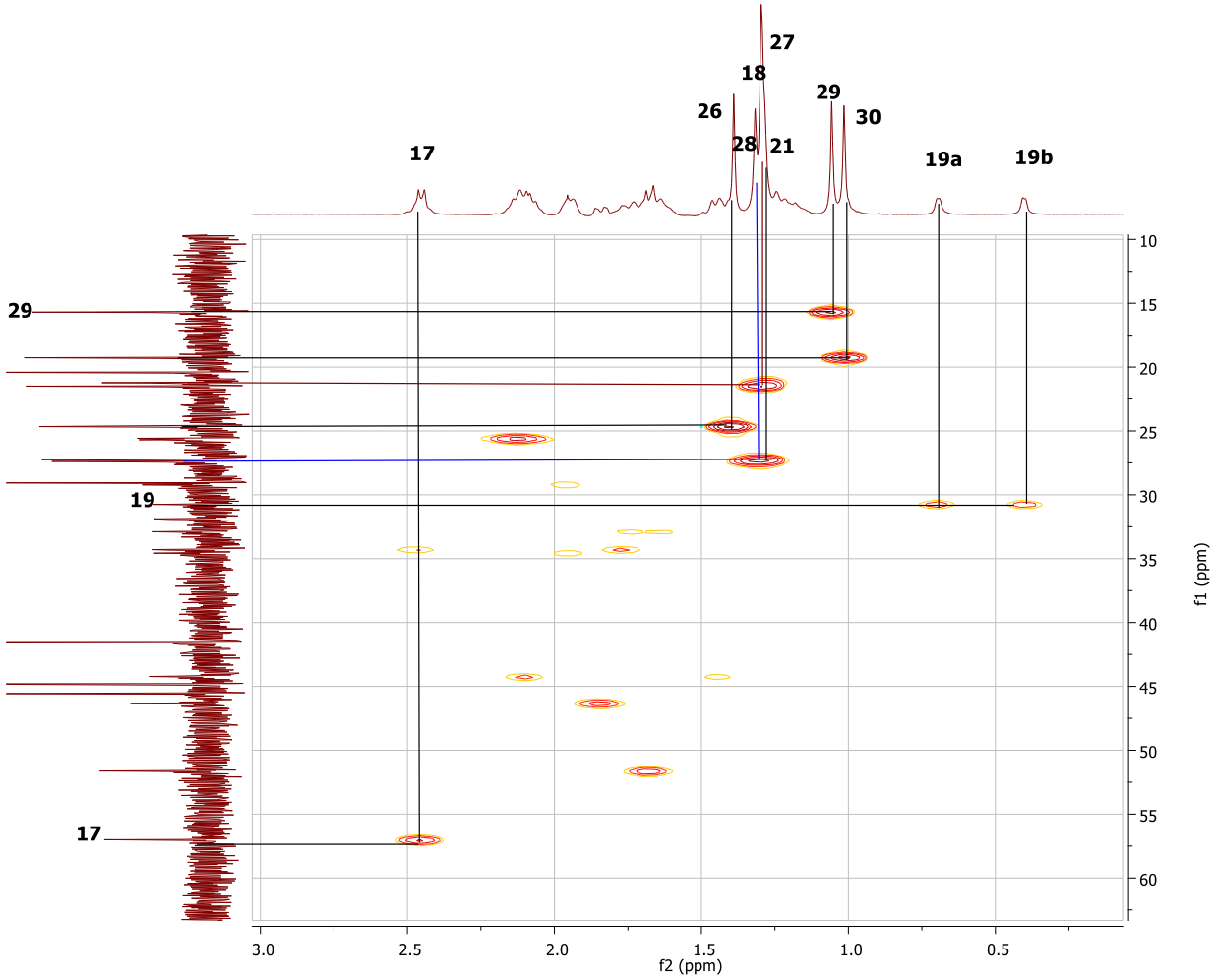
Spectrum 3.1. ^{1}H Spectrum of DC-AST VII (400 MHz, D_2O)



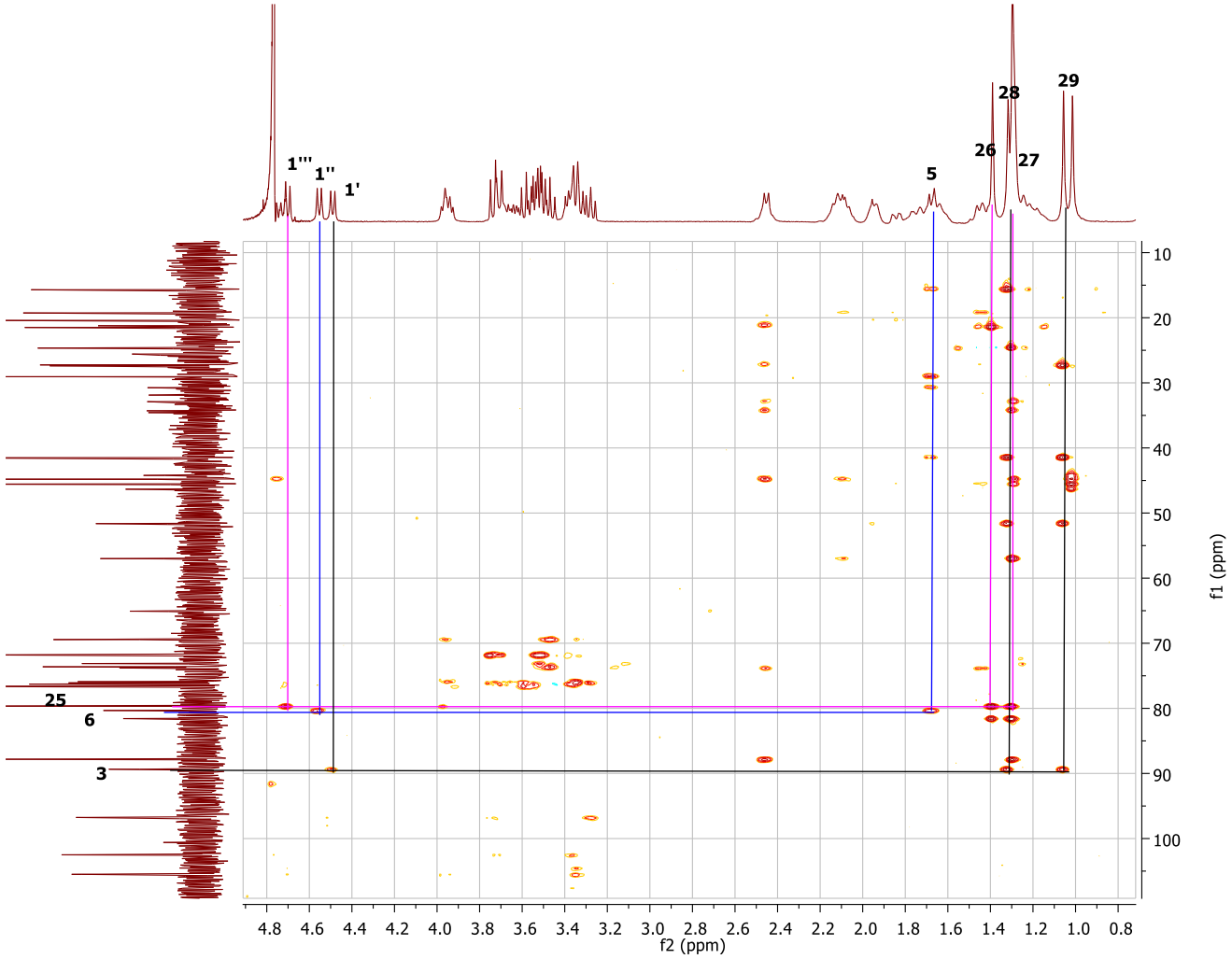
Spectrum 3.2. ^{13}C Spectrum of DC-AST VII (100 MHz, D_2O)



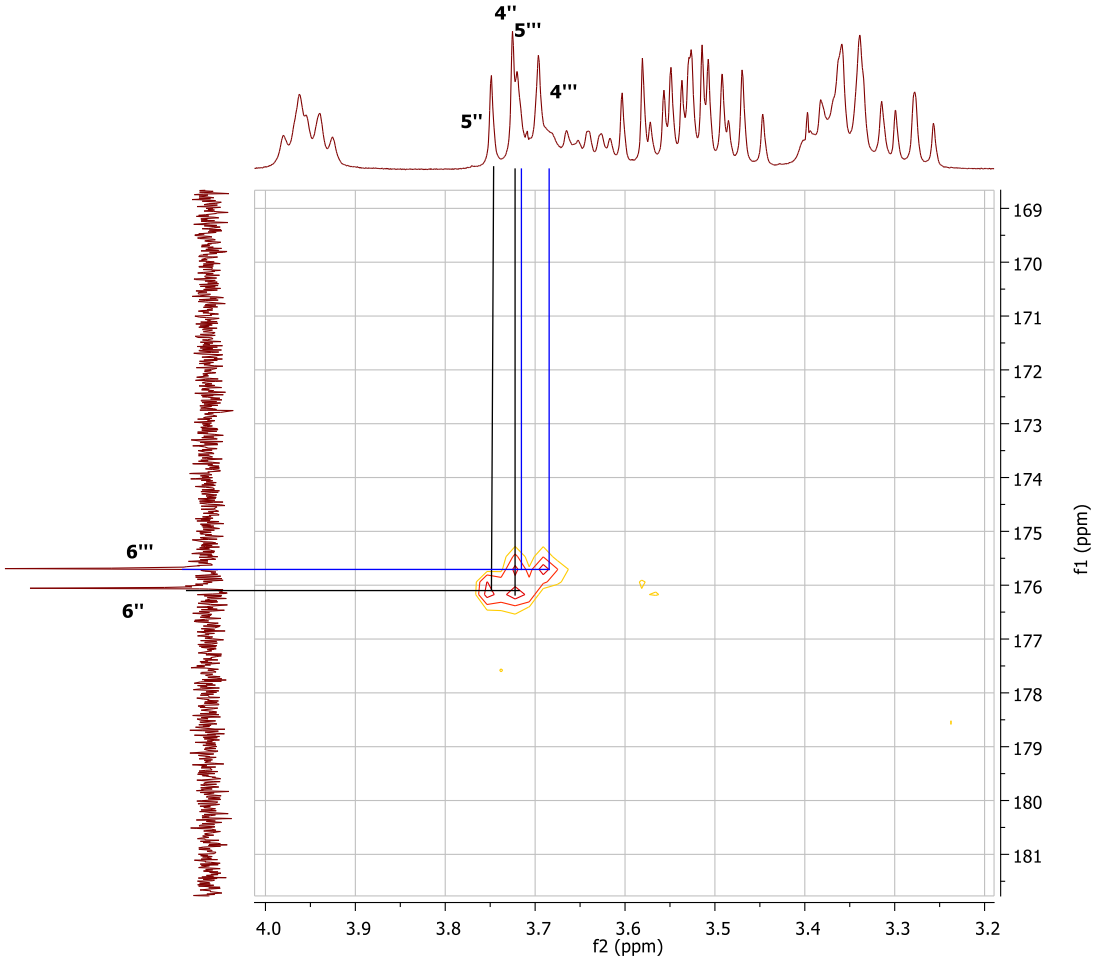
Spectrum 3.3. HSQC Spectrum of DC-AST VII



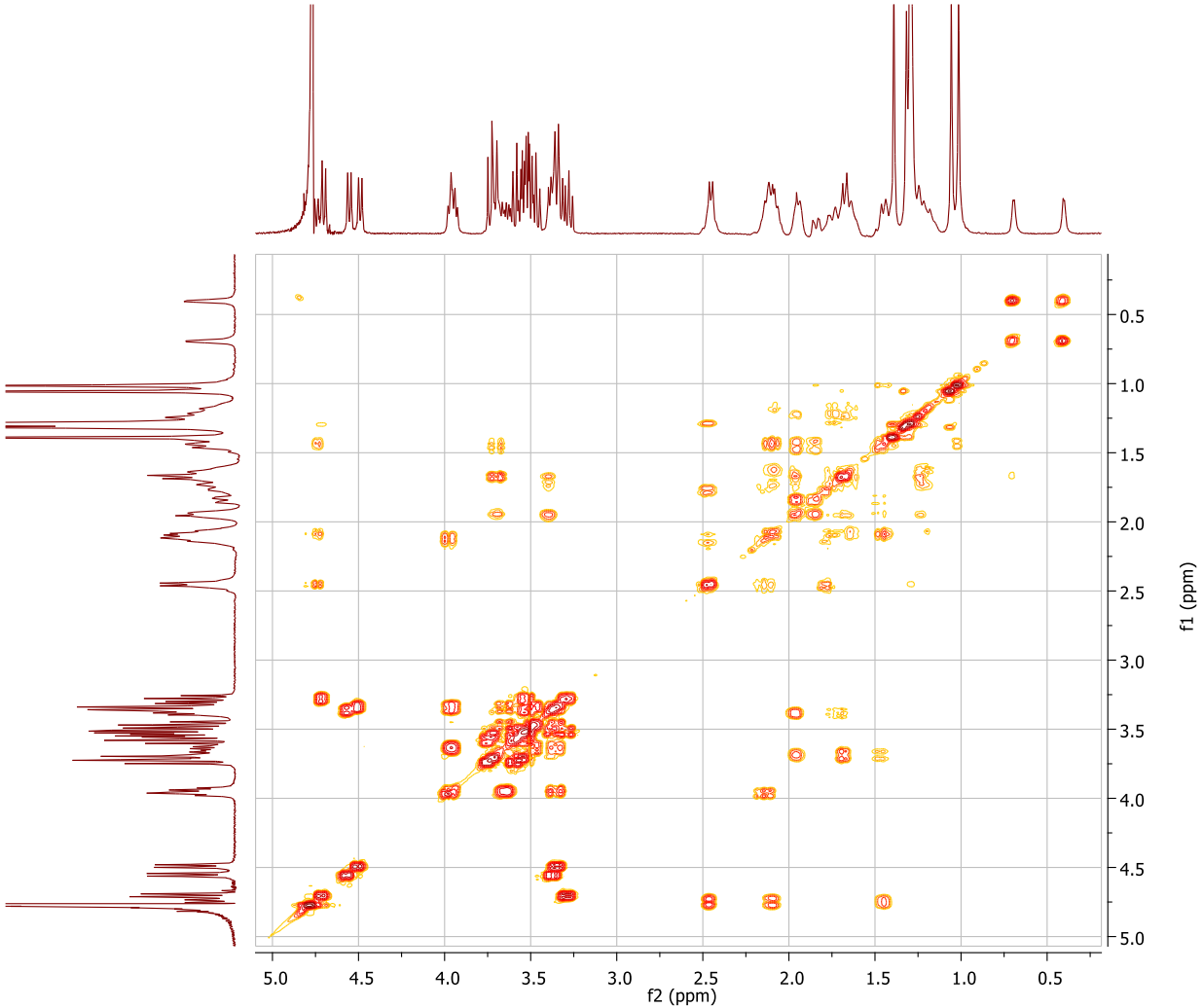
Spectrum 3.4. HSQC Spectrum of DC-AST VII



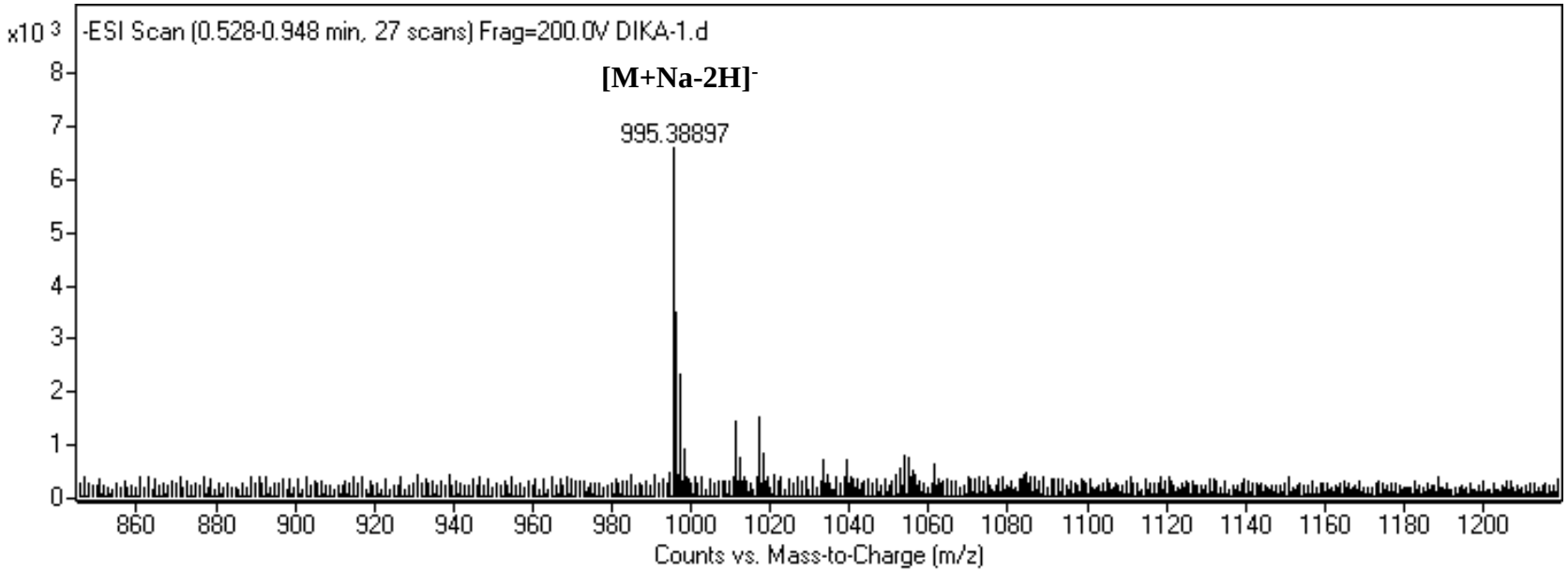
Spectrum 3.5. HMBC Spectrum of DC-AST VII



Spectrum 3.6. HMBC Spectrum of DC-AST VII



Spectrum 3.7. COSY Spectrum of DC-AST VII



Spectrum 3.8. HR-ESI-MS Spectrum of DC-AST VII (Negative mode)

3.2. Structural Identification of DAC-AST VII

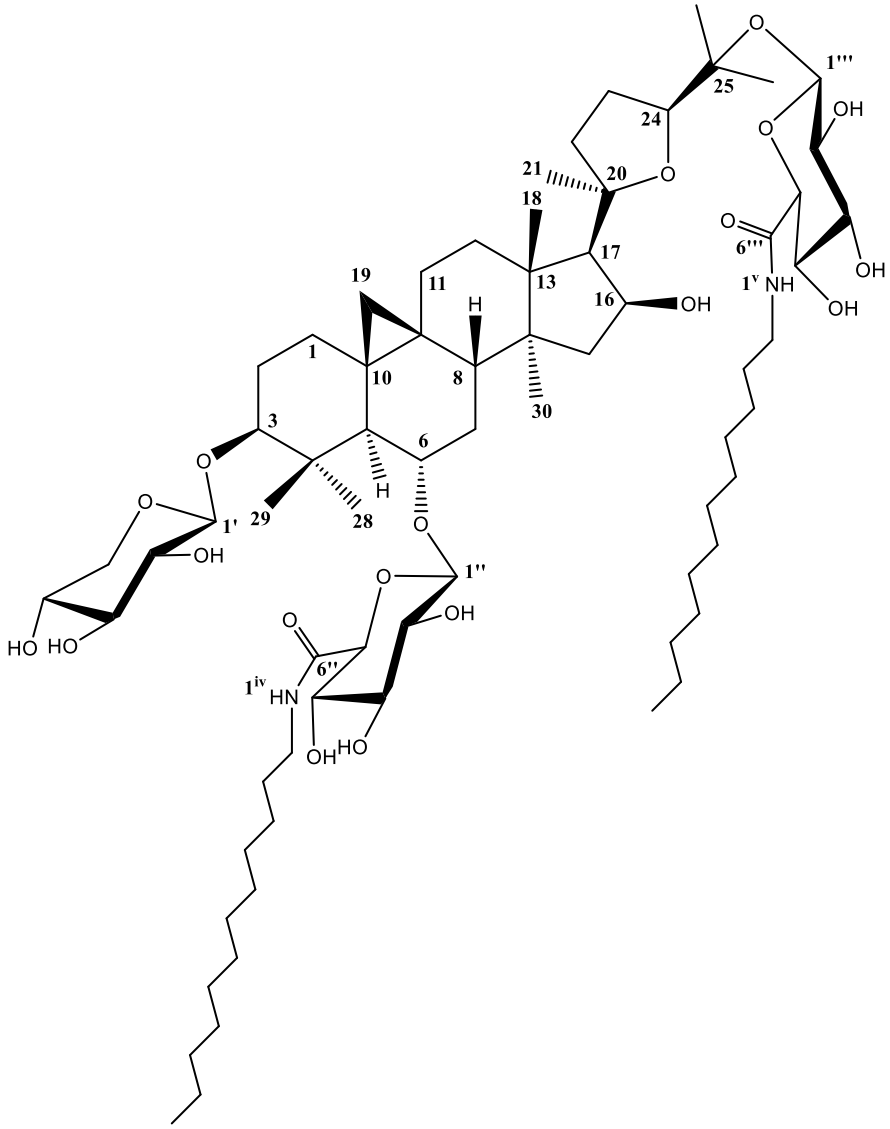


Figure 3.2. Chemical Structure of Dodecylamine Conjugated AST VII (DAC-AST VII)

IUPAC name:

(2S,3S,4S,5R,6S)-N-dodeccyl-6-((2-((2S,5R)-5-((2aR,3R,4S,5aS,5bS,7S,7aR,9S,11aR,12aS)-7-(((2R,3R,4S,5S,6S)-6-(dodecylcarbamoyl)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)oxy)-4-hydroxy-2a,5a,8,8-tetramethyl-9-(((2S,3R,4S,5R)-3,4,5-trihydroxytetrahydro-2H-pyran-2-yl)oxy)tetradecahydro-1H,12H-cyclopenta[a]cyclopropa[e]phenanthren-3-yl)-5-

methyltetrahydrofuran-2-yl)propan-2-yl)oxy)-3,4,5-trihydroxytetrahydro-2H-pyran-2-carboxamide

Molecular Formula: $C_{71}H_{124}N_2O_{19}$

Exact Mass: 1308.88

A major ion peak was observed at m/z 1131.89196 $[M+Na]^+$ indicating the molecular formula as $C_{71}H_{124}N_2O_{19}$ (calculated 1331.88 for $C_{71}H_{124}N_2O_{19}Na$) in the HR-ESI-MS spectrum (positive mode) of DAC-AST VII. ^{1}H NMR spectrum of DAC-AST VII displayed signals for a cyclopropane methylene at δ 0.61 and 0.25 (each 1H, d, *J*= 3.8, 4.2 Hz) and seven tertiary methyl groups at 1.99 (3H, s), 1.64 (3H, s), 1.44 (3H, s), 1.42 (3H, s), 1.37 (3H, s), 1.32 (3H, s), 1.12 (3H, s). The terminal methyl groups of aliphatic carbon chains were resonated at δ 0.88 (H-13^{v} and H-13^{iv}). Three anomeric protons and carbons were resonated at δ 5.12, d, (*J*=7.8 Hz, 1H), δ 99.1 d; δ 4.95, d, (*J*=7.8 Hz, 1H), δ 105.2 d; δ 4.86, d, (*J*=7.4 Hz, 1H), δ 108.2 d, respectively. COSY, HSQC and HMBC spectra led to identify other H/C signals of aglycone and sugar moieties. The HMBC correlations between the proton signals at δ 4.86 (H-1'), δ 4.95 (H-1''), δ 5.12 (H-1''') and the carbon resonance at δ 88.9 (C-3), δ 79.5 (C-6), δ 79.7 (C-25) were similar with signals of DC-AST VII. Dodecylamine conjugation into C-6 at the glucuronic acids resulted in 4 ppm upfield shift at C-6'' [from δ 176.1 to δ 171.5] and C-6''' [from δ 175.7 to δ 171.4] compare to DC-AST VII. The ^{1}H NMR spectrum demonstrated that the proton signals of N-H were resonated at δ 8.13, t (*J*=6.2 Hz) and δ 8.09, t (*J*=6.1 Hz). It was indicated that two free dodecylamines were conjugated into glucuronic acids via amide bond formation. The dodecylamine conjugations were also verified by C-H interaction in HMBC spectrum. Strong key correlation peaks were observed between the carbon signals at δ 171.5 (C-6'') and δ 171.4 (C-6''') and the proton signals of aliphatic carbon chains at δ 3.46 (H-2^{iv}), δ 3.64 (H-2^{v}), Two bond correlation peaks were displayed between the carbon signals at δ 171.5 (C-6''), δ 171.4 (C-6''') and the proton signals of at δ 4.34 (H-5''), δ 4.35 (H-5''') on the sugar moieties and also the proton signals of at δ 8.19 (H-1^{iv}), δ 8.13 (H-1^{v}) on the aliphatic carbon chains, respectively. The structure of DAC-AST VII was established as 3-*O*-β-D-xylopyronosyl-6,25-di-*O*-(6'',6'''-di-dodecylamino)-β-D-glucuronopyronosyl-20(*R*),24(*S*)-epoksi-3β-6α-16β-25-tetrahydroxycycloartane.

Table 3.2. The ^{13}C and ^{1}H NMR data of DAC-AST VII (100/400 MHz, δ ppm, Pyridine-d5)

H/C	δ_C (ppm)	δ_H (ppm), *J* (Hz)
1	32.7 t	1.56 m, 1.80 m
2	30.2 t	1.73 m, 1.91 m
3	88.9 d	3.53 dd (11.8, 4.4)
4	43.2 s	
5	52.9 d	1.88 m
6	79.5 d	3.83 m
7	34.7 t	1.84 m, 2.15 dd (8.5, 3.5)
8	45.9 d	2.01 m
9	21.5 s	
10	29.5 s	
11	26.7 t	1.95 m, 2.33 m
12	33.9 t	1.56 m, 1.70 m
13	46.8 s	
14	45.9 s	
15	46.7 t	1.77 m, 2.26 dd (12.3, 7.9)
16	74.1 d	4.99
17	58.6 d	2.53 m
18	21.6 q	1.42 s
19	28.9 t	0.25 d (4.1), 0.61 d (3.9)
20	87.7 s	
21	27.8 q	1.32 s
22	35.7 t	1.64 m, 2.78 t (9.9)
23	26.5 t	1.95 m, 2.38 m
24	82.5 d	3.95 dd (8.5, 6.1)
25	79.7 s	
26	25.4 q	1.64 s
27	23.5 q	1.44 s
28	28.2 q	1.99 s
29	17.1 q	1.37 s
30	20.6 q	1.12 s
1'	108.2 d	4.86 d (7.4)
2'	74.9 d	4.04 dd
3'	79 d	4.16 t
4'	71 d	4.21 m
5'	67.6 t	3.71 t, 4.37 dd
1''	105.2 d	4.95 d (7.8)
2''	75.4 d	4.04 dd
3''	78.9 d	4.25 dd
4''	74.2 d	4.27 m
5''	76.6 d*	4.34 dd
6''	171.5 s	
1'''	99.1 d	5.12 d (7.8)
2'''	78.9 d	3.99 d
3'''	78.3 d	4.21 m

(cont. on next page)

Table 3.2. (cont.)

H/C	δ_C (ppm)	δ_H (ppm), *J* (Hz)
4^{III}	74.4 d	4.20 m
5^{III}	76.4 d*	4.35 dd
6^{III}	171.4 s	
1^{IV}		8.09 t (6.1)
2^{IV}	39.9 t	3.46 m
3^{IV}	27.8 t	1.65 m
4^{IV}-11^{IV}	30-31 t	1.62-1.82 m
12^{IV}	32.7 t	1.29 m
13^{IV}	14.6 q	0.88 s
1^{V}		8.13 t (6.2)
2^{V}	39.8 t	3.64 m
3^{V}-11^{V}	30-31 t	1.62-1.82 m
12^{V}	32.7 t	1.29 m
13^{V}	14.6 q	0.88 s

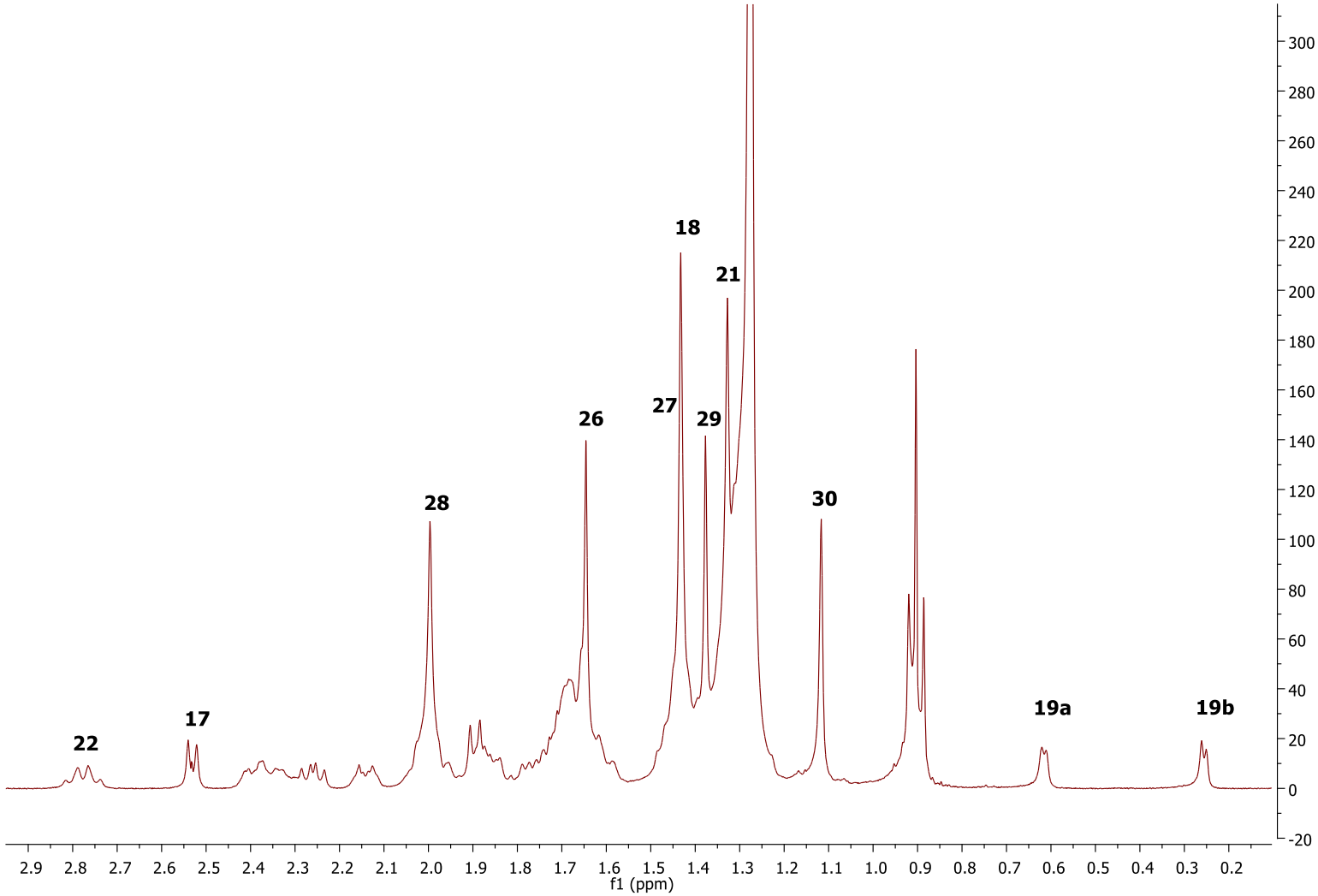
*****: changeable

3.3. Evaluation of Immunomodulatory Activities of AST VII and its Analogs

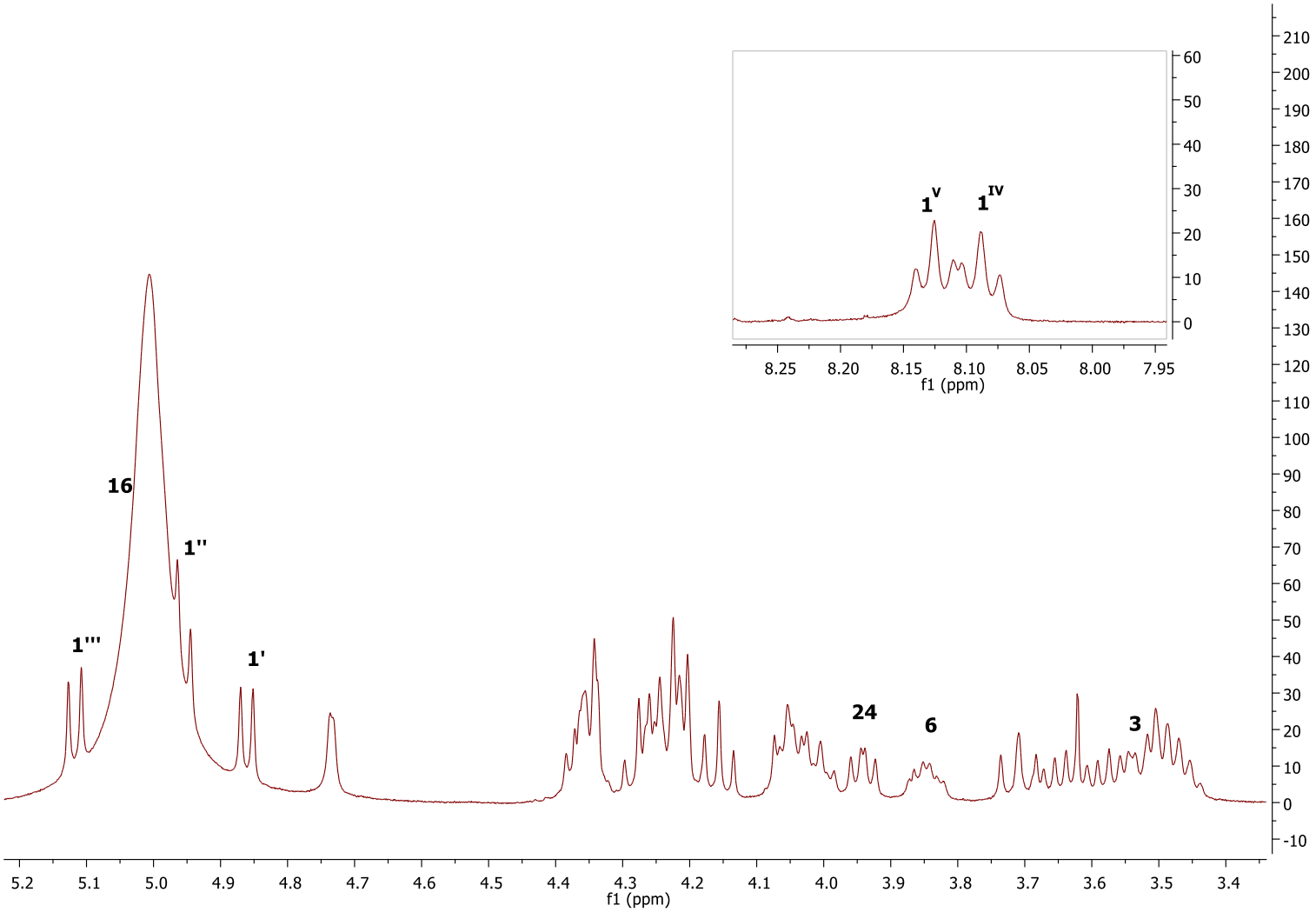
3.3.1. Cytokine Release Study on Human Whole Blood

Whole blood (WB) assay is a simple and effective approach to evaluate immunomodulatory activities of the test compounds as it contains broad immune cells, such as. T cells, B cells, NK cells, monocytes and granulocytes. Cytokines, indicators of cell mediated immune response, regulate the initiation and maintenance of the immune response.[114] The type of immune response (Th1/Th2/Th17) induced by AST VII and its analogs was investigated based on their cytokine release profiles (IL-2, IFN-γ, IL-17A, IL-1β, TNF-α, IL-4 etc.) on human WB.

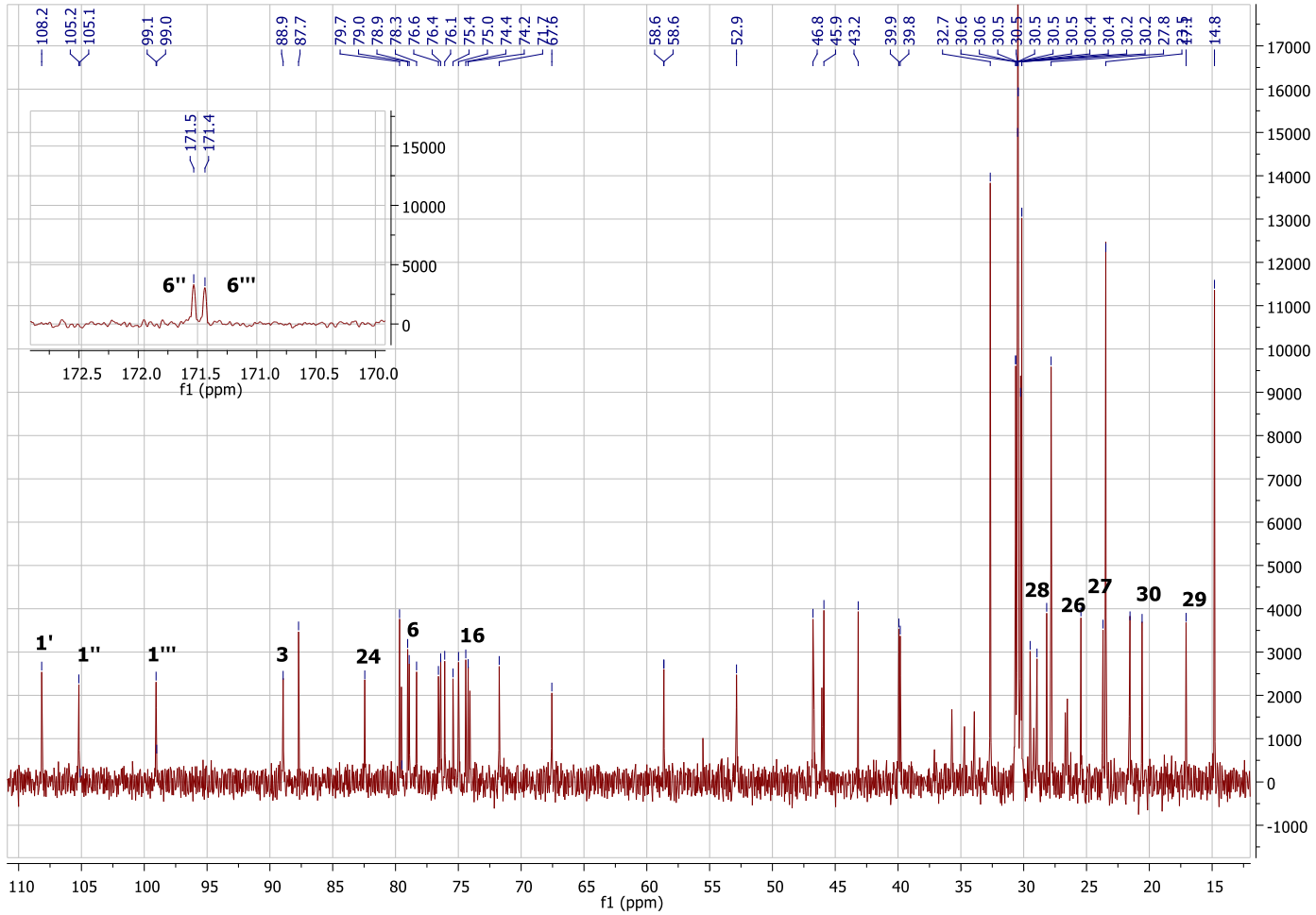
Yeşilada and his co-worker's modified WB protocol was followed in this study. Human whole blood was diluted 1/10 and 1/20 in R5 medium and stimulated with PMA (phorbol 12-myristate 13-acetate) (50 ng/mL) and ionomycin (400 ng/mL). PMA (protein kinase C activator) is commonly used with ionomycin (calcium ionophore) to



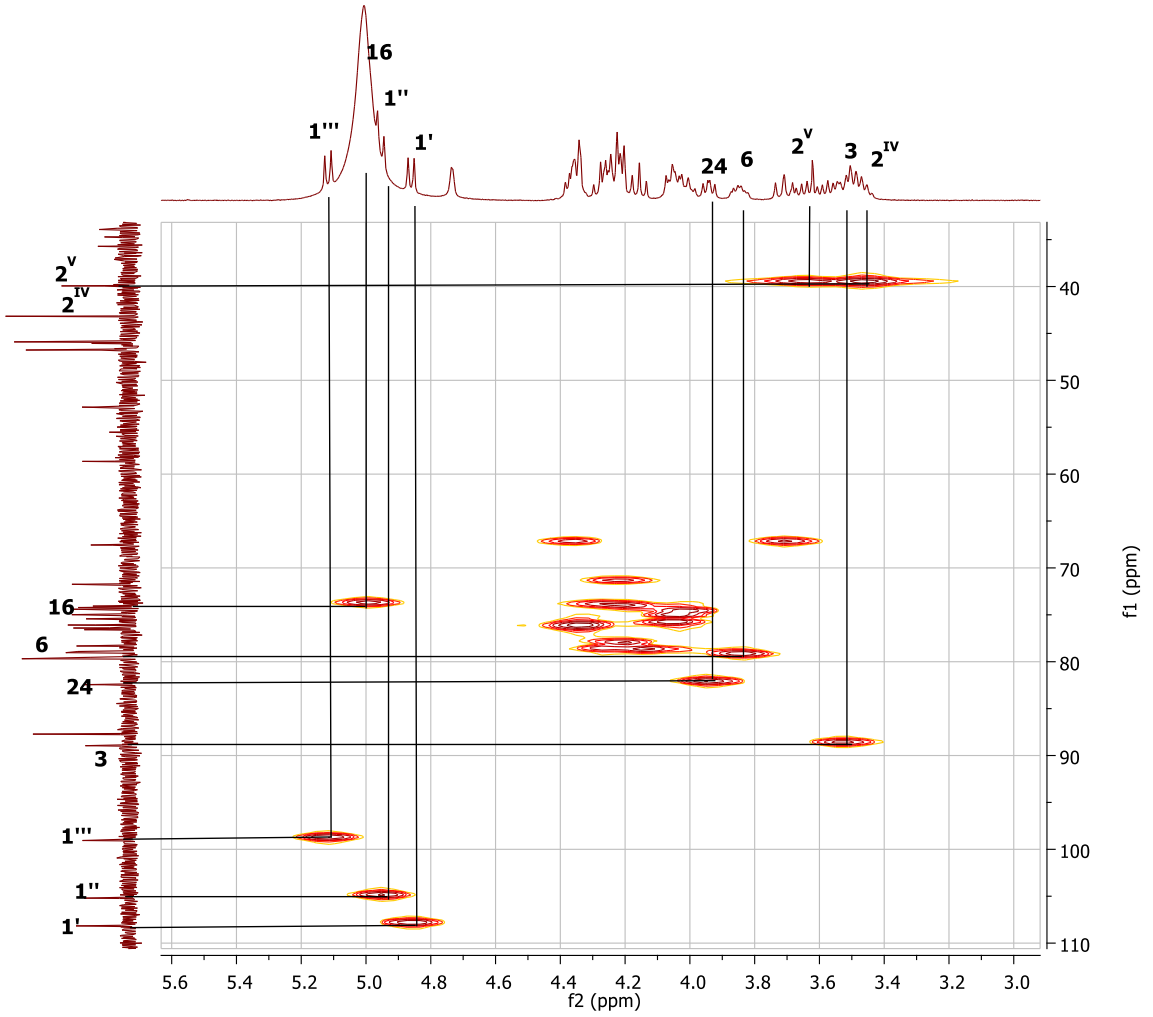
Spectrum 3.9. ^{1}H Spectrum of DAC-AST VII (400 MHz, d5-pyridine)



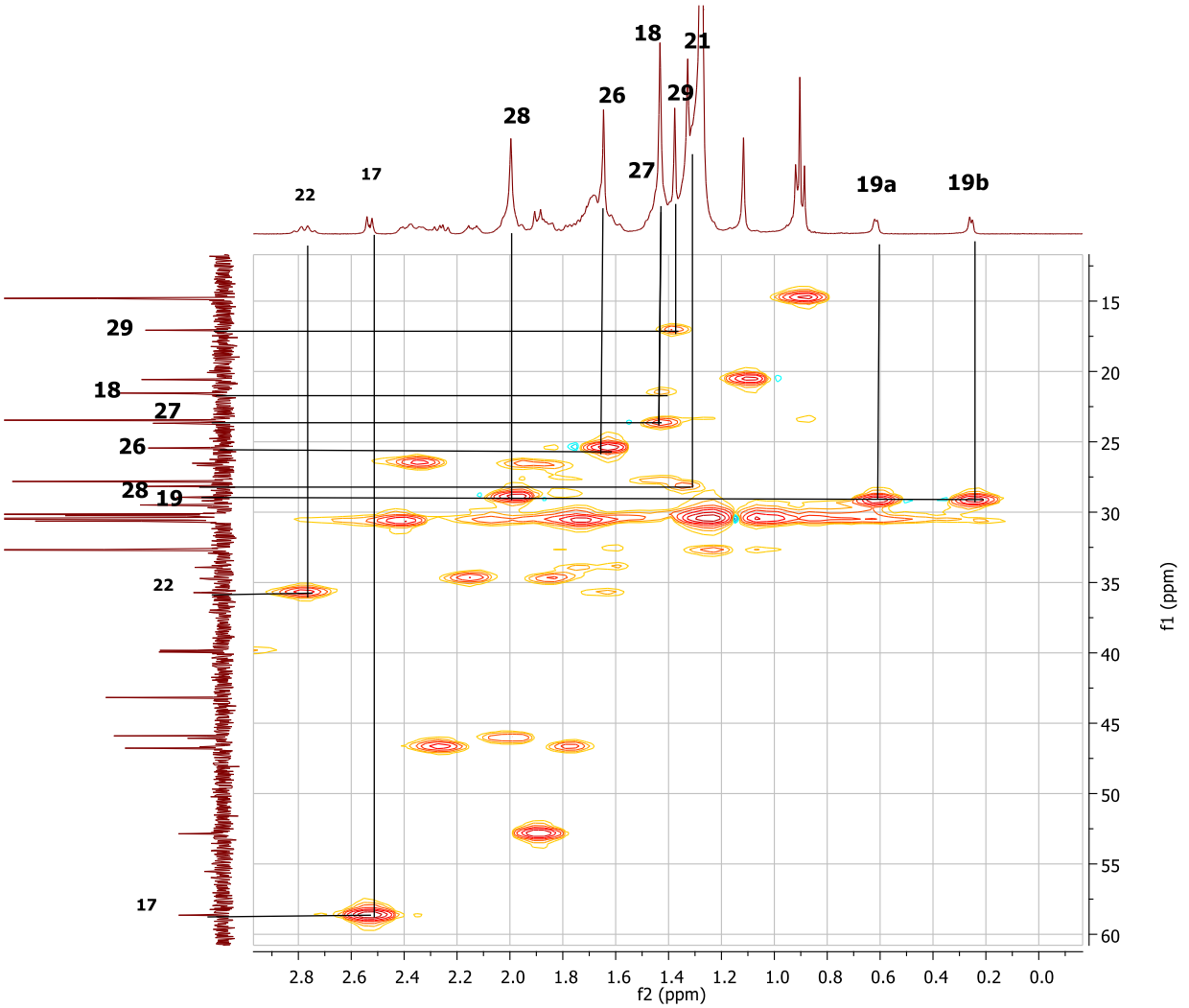
Spectrum 3.10. ^{1}H Spectrum of DAC-AST VII (400 MHz, d5-pyridine)



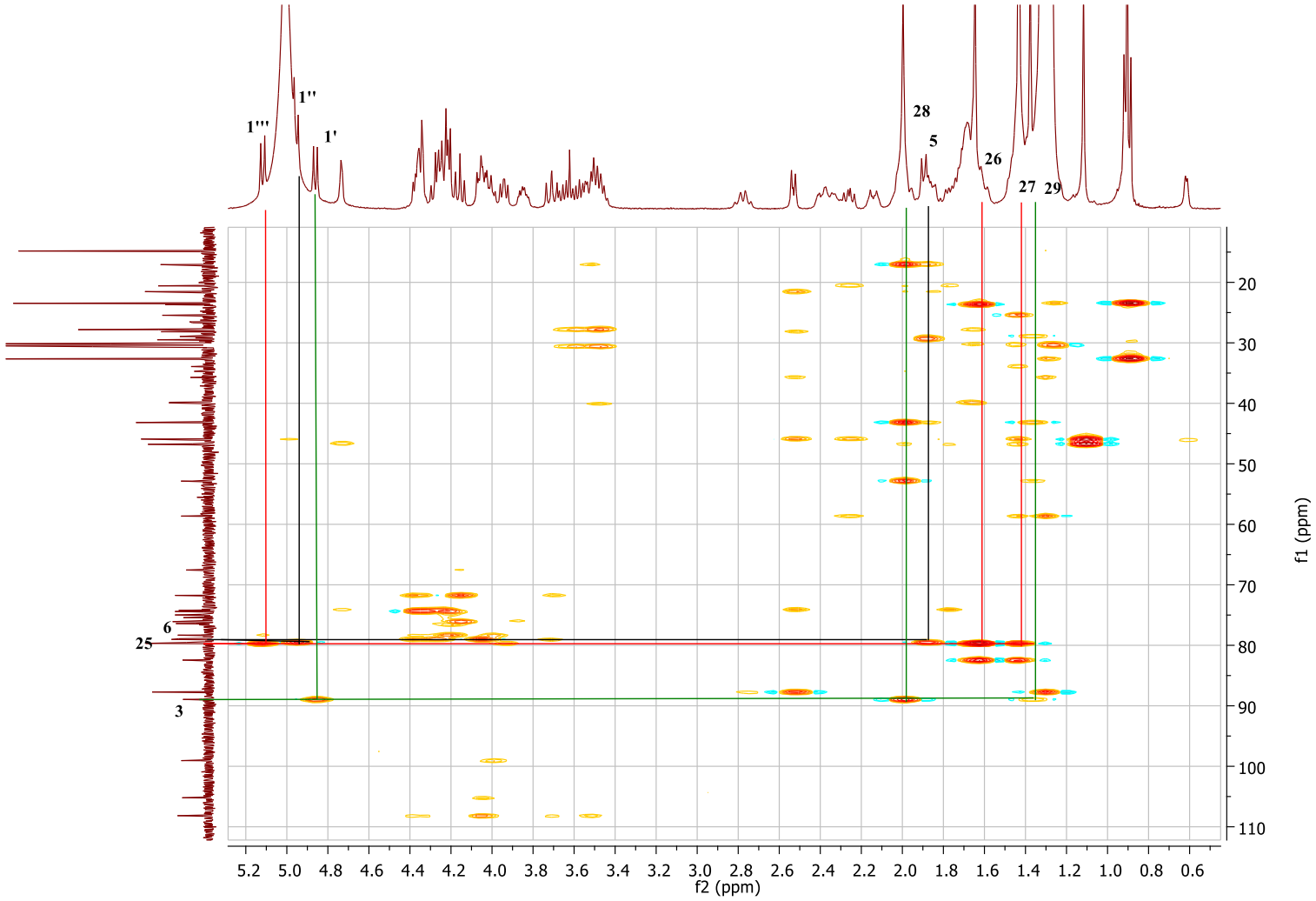
Spectrum 3.11. ^{13}C Spectrum of DAC-AST VII (100 MHz, d5-pyridine)



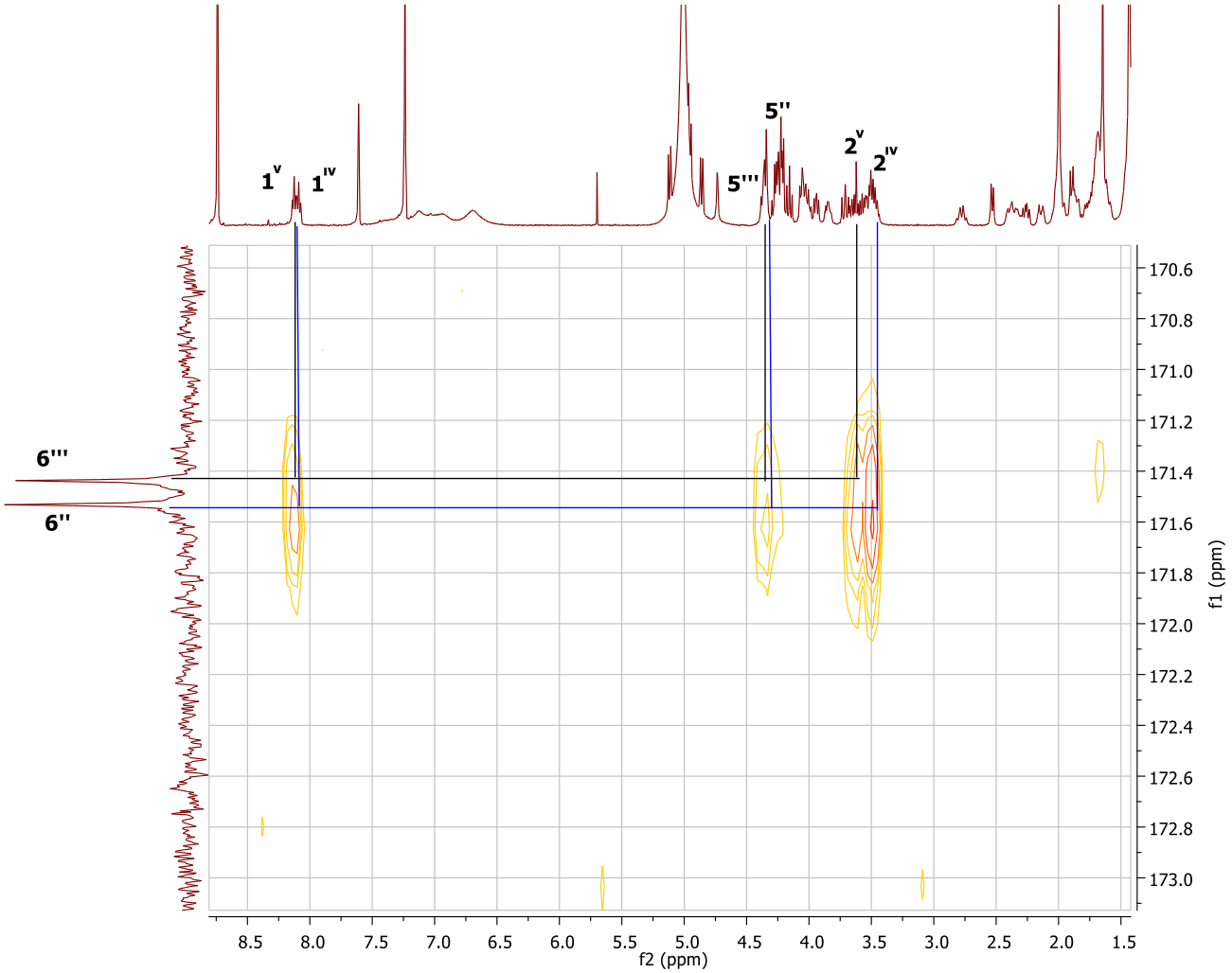
Spectrum 3.12. HSQC Spectrum of DAC-AST VII



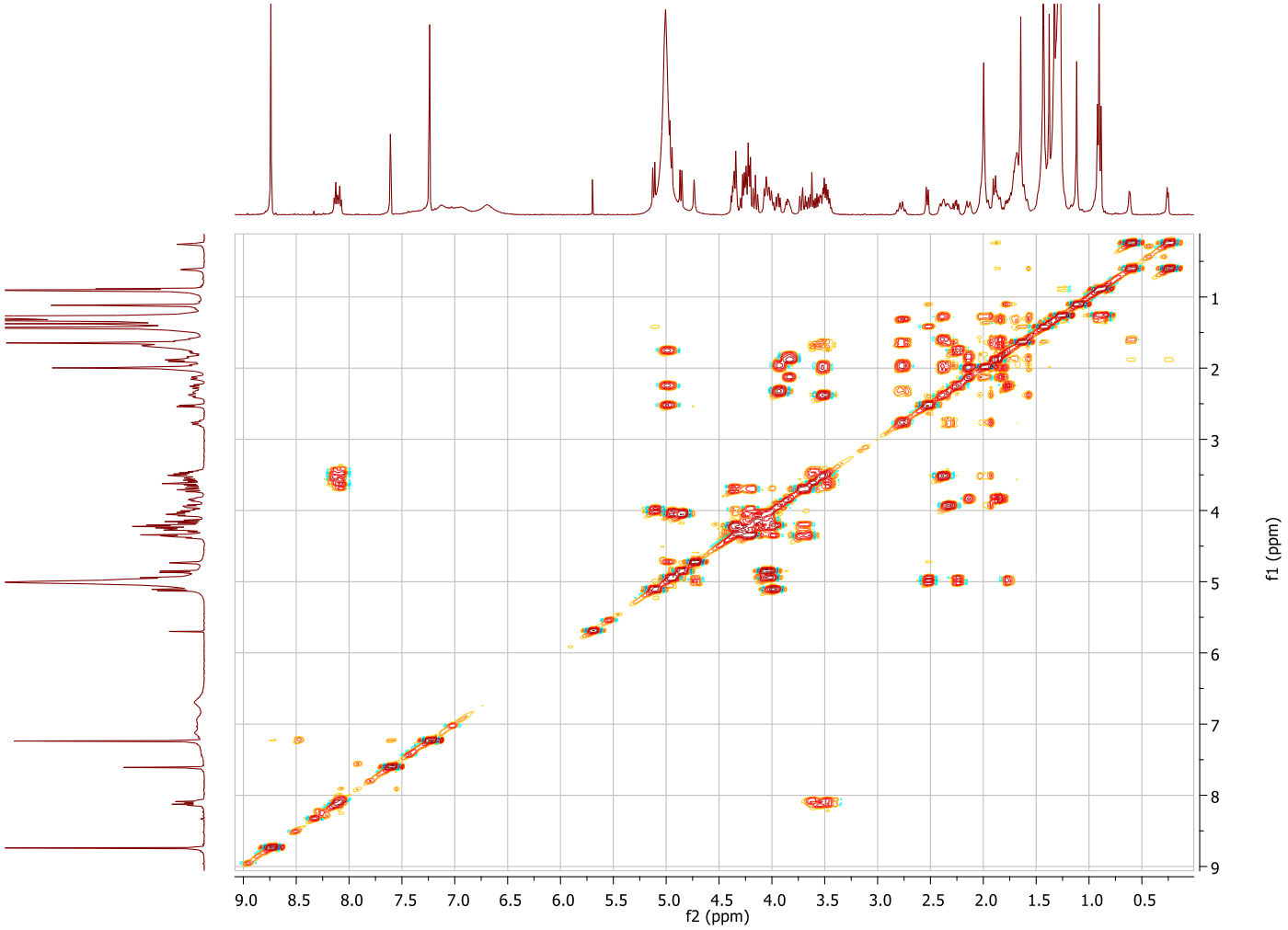
Spectrum 3.13. HSQC Spectrum of DAC-AST VII



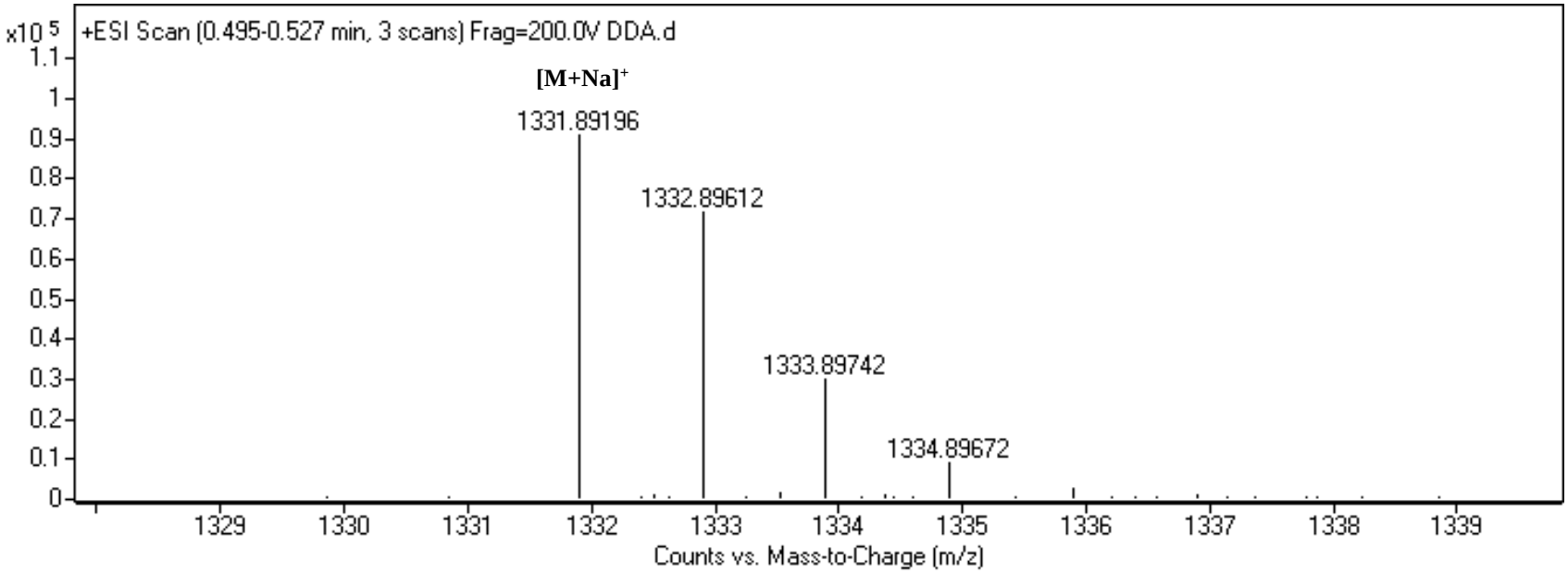
Spectrum 3.14. HMBC Spectrum of DAC-AST VII



Spectrum 3.15. HMBC Spectrum of DAC-AST VII



Spectrum 3.16. COSY Spectrum of DAC-AST VII



Spectrum 3.17. HR-ESI-MS Spectrum of DAC-AST VII (positive mode)

activate T cells by inducing NF-κB and NFAT transcription factors successively leading to production of cytokines especially IL-2.[115]

In the first experiment set, PMA-ionomycin treatment in 1/10 diluted whole blood resulted in insignificant induction of cytokines compared to untreated cells. The fail in the experiment was thought to be originating from high cell population. Interestingly, in this experiment set, IL-1β and IL-17A cytokines were remarkably stimulated with co-treatment of AST VII, DC-AST VII and DAC-AST VII with PMA+ionomycin in dose dependent manner, whereas IL-2, IFN-γ, TNF–α and IL-4 productions were not altered following treatments (Figure 3.3, Figure 3.4.).

IL-1β is a pro-inflammatory cytokine produced by blood monocytes, tissue macrophages, dendritic cells, B lymphocytes, natural killer cells, fibroblast and epithelial cells.[116] IL-1 regulates dendritic cell activation, cytokine production and differentiation of naive T cells.[115] Moreover, IL-1 enables T cell proliferation by upregulating IL-2R (IL-2 receptor) expression and activates several signaling pathways such as NF-κB, phosphoinositide 3-kinase[115]. As shown in Figure 3.3., PMA-ionomycin cytokine level was close to the untreated group. However, IL-1β levels were substantially enhanced with the stimulation of AST VII, DC-AST VII and DAC-AST VII, indicated by IL-1β titers: AST VII (4 μg/mL) 2.24 fold ($p < 0.01$), AST VII (8 μg/mL) 1.79 fold ($p < 0.01$), AST VII (16 μg/mL) 1.55 fold ($p < 0.05$), AST VII (32 μg/mL) 1.92 fold ($p < 0.05$), DC-AST VII (2 μg/mL) 1.96 fold ($p < 0.05$), DC-AST VII (4 μg/mL) 2.25 fold ($p < 0.001$), DC-AST VII (8 μg/mL) 2.52 fold ($p < 0.01$), DC-AST VII (16 μg/mL) 1.76 fold ($p < 0.05$), DC-AST VII (32 μg/mL) 2.4 fold ($p < 0.01$), DAC-AST VII (2 μg/mL) 2.79 fold ($p < 0.01$), DAC-AST VII (4 μg/mL) 2.95 fold ($p < 0.001$), DAC-AST VII (8 μg/mL) 3.16 fold ($p < 0.01$), DAC-AST VII (16 μg/mL) 3.32 fold ($p < 0.001$), DAC-AST VII (32 μg/mL) 3.21 fold ($p < 0.01$) compare to PMA-Ionomycin.

IL-17A is also one of the pro-inflammatory cytokines produced by Th17 cells, γδ T cells, innate Th17 cells. IL-17A has immunoregulatory properties promoting production of cytokines and chemokines to recruit neutrophils and macrophages to the inflammation site. Moreover, it has a protective role against bacterial and fungal infections.[117] In the experiment set 1, IL-17A levels were significantly increased by QS-21 and AST VII treatments as indicated in Figure 3.4.: QS-21 (2 μg/mL) 5.32 fold ($p < 0.001$), QS-21 (4 μg/mL) 5.84 fold ($p < 0.001$), QS-21 (8 μg/mL) 5.49 fold ($p < 0.001$),

QS-21 (16 μg/mL) 5.12 fold ($p < 0.01$), QS-21 (32 μg/mL) 4.6 fold ($p < 0.01$), AST VII (2 μg/mL) 5.05 fold ($p < 0.01$), AST VII (4 μg/mL) 4.31 fold ($p < 0.01$), AST VII (8 μg/mL) 4.58 fold ($p < 0.01$), AST VII (16 μg/mL) 4.79 fold ($p < 0.05$), AST VII (32 μg/mL) 5.03 fold ($p < 0.01$), DC-AST VII (2 μg/mL) 5.69 fold ($p < 0.05$), DAC-AST VII (16 μg/mL) 3.15 fold ($p < 0.05$) compared to PMA-Ionomycin.

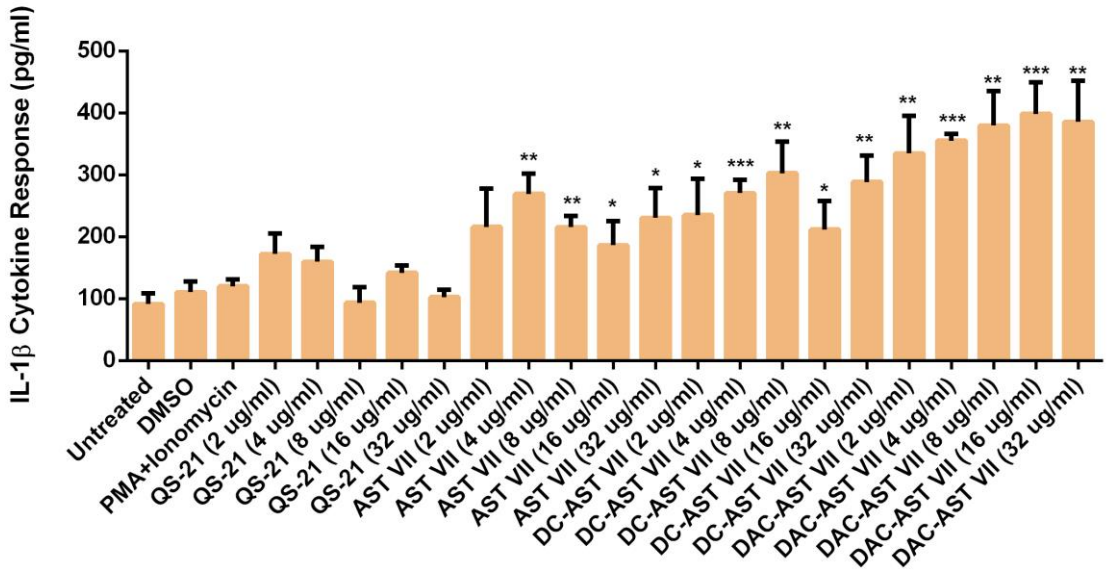


Figure 3.3. Experiment set 1: IL-1β cytokine concentration (pg/mL) in human whole blood co-stimulated with different saponin molecules and PMA/ ionomycin. All groups were performed in triplicate. The significant difference is defined between sample groups and PMA/ionomycin: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

IL-1β helps naive T cells to differentiate to Th17 cell and promotes IL-17 producing memory $CD4^+$ T cells.[118] AST VII significantly promoted IL-17A cytokine production in the presence of mid level of IL-1β in the microenvironment whereas analogs of AST-VII were not able to show such an effect on IL-17A secretion except for DC-AST VII 2 μg/mL and DAC-AST VII 16 μg/mL. Kryczek et al. have demonstrated that IL-1 is a profound promoter of $IL\text{-}17^+$ T cell differentiation with TGF-β.[119] Nalbantsoy et al. revealed that AST VII in the presence of LPS was able to stimulate TGF-β production.[56] Based on these results, it can be stated that AST VII drives IL-17A production in the presence of IL-1β and TGF-β.

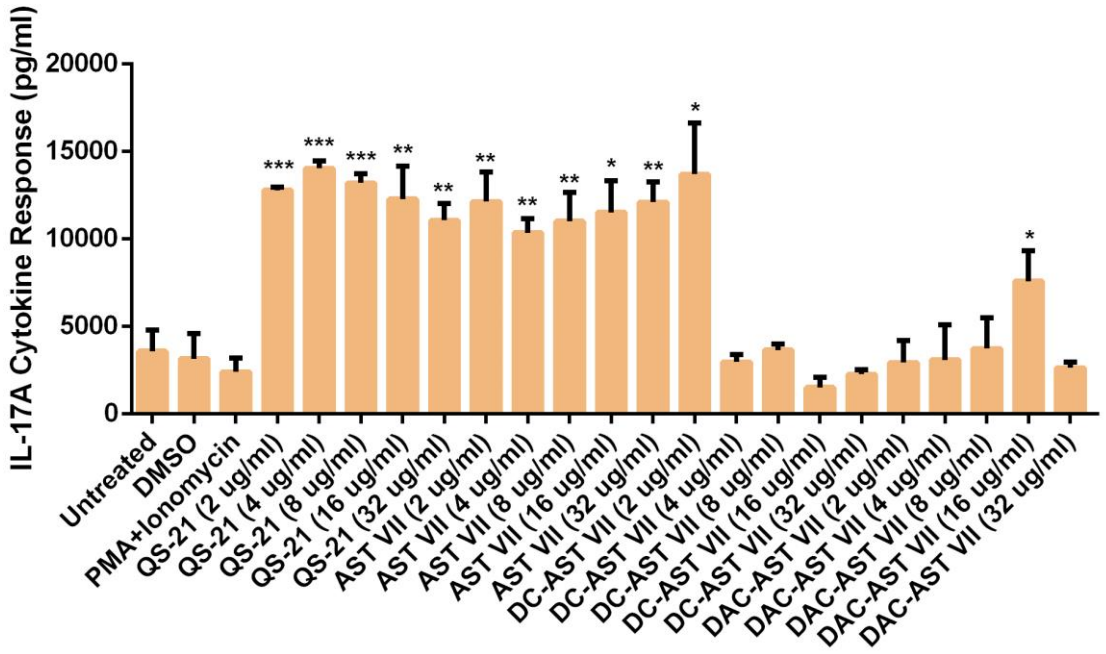


Figure 3.4. Experiment set 1: IL-17Acytokine concentration (pg/mL) in human whole blood co-stimulated with different saponin molecules and PMA/ ionomycin. All groups were performed in triplicate. The significant difference is defined between sample groups and PMA/ionomycin: *p < 0.05, **p < 0.01, ***p < 0.001

In the second experiment set, 1/20 diluted whole blood was used. PMA-ionomycin alone considerably induced productions of IL-2, IFN-γ and moderately TNF-α. IL-1β was not reached to higher titer levels following AST VII and DC-AST VII co-treatment with PMA+ionomycin; however, DAC-AST VII stimulation significantly boosted IL-1β. IFN-γ and IL-2 cytokines were suppressed with administration of AST VII, DC-AST VII, DAC-AST VII compared to PMA-ionomycin. IL-17A response was not observed as in experiment set 1.

As shown in Figure 3.5., only DAC-AST VII significantly stimulated IL-1β production: DAC-AST VII (2 μg/mL) 2.30 fold ($p < 0.05$), DAC-AST VII (4 μg/mL) 2.55 fold ($p < 0.01$), DAC-AST VII (8 μg/mL) 3.33 fold ($p < 0.001$), DAC-AST VII (16 μg/mL) 4.2 fold ($p < 0.001$), DAC-AST VII (32 μg/mL) 3.64 fold ($p < 0.01$) compared to PMA- Ionomycin.

There was no appreciable difference in IL-1β levels between AST VII and DC-AST VII treated groups. On the other hand, increasing the lipophilicity by conjugation of dodecylamines in the structure of AST VII (DAC-AST VII) strongly boosted IL-1β

production compared to AST VII. Calculated partition coefficients (clogP) clearly demonstrates the lipophilic order of AST compounds [DAC-AST VII (clogP: 11.41) > AST VII (clogP: 1.29) > DC-AST VII (clogP: 0.34)], implying importance of polarity for IL-1β titers as (Figure 3.5.). Since fatty acid substances including ceramides are inducers of IL-1β production[120], one should also consider that hydrolysis of the amide bond affording dodecylamine might be one of the reasons for such a significant effect. A detailed literature search returned with no report of dodecylamine/IL-1β connection. However, in our subsequent studies, possible effect of dodecylamine by itself on IL-1β release will be evaluated to clarify this important issue.

IL-2 is one of the Th1 cytokines and stated as "T cell growth factor". It is produced by $CD4^+$ T, $CD8^+$ T, NK, NKT and dendritic cells.[116] IL-2 promotes the clonal expansion and differentiation of T cells, specifically differentiation of naive $CD8^+$ T cells to effector and memory T cells.[117] Sixty percent of $CD4^+$ T cells secrets IL-2 following non-specific stimulation such as PMA-ionomycin.[117]

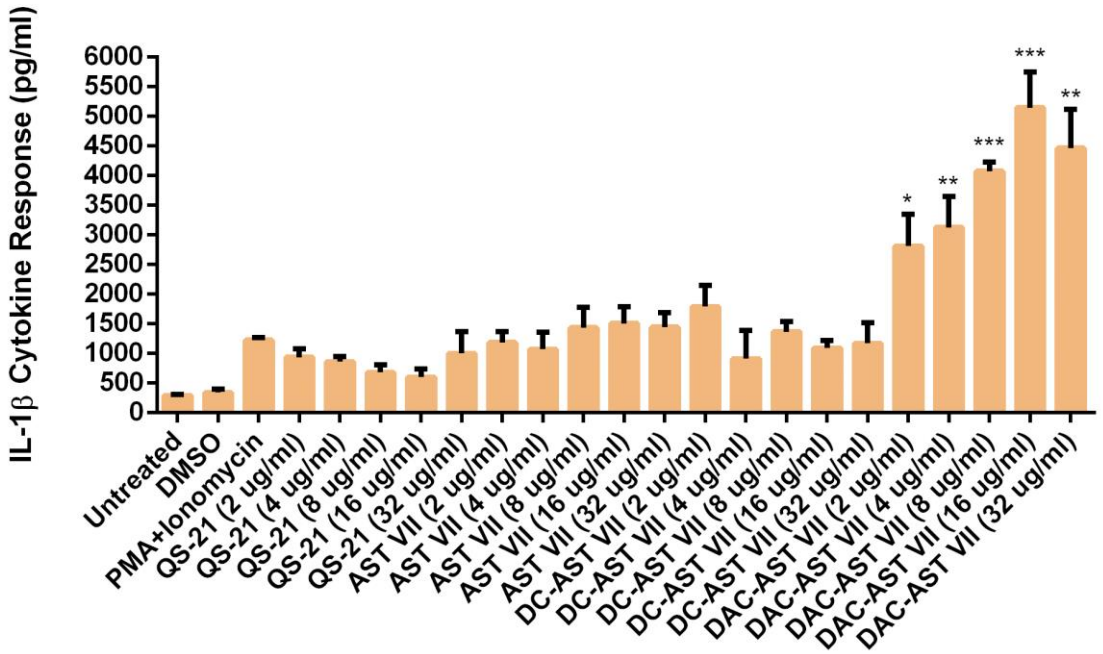


Figure 3.5. Experiment set 2: IL-1β cytokine concentration (pg/mL) in human whole blood stimulated with PMA/ionomycin alone or PMA/ionomycin + different saponin molecules. All groups were performed in triplicate. The significant difference is defined between sample groups and PMA/ionomycin: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

As shown in Figure 3.6., PMA-ionomycin significantly induced IL-2 production but all saponin treatments in the presence of PMA-ionomycin suppressed IL-2: QS-21 (2 μg/mL) 2.35 fold ($p < 0.001$), QS-21 (4 μg/mL) 2.47 fold ($p < 0.001$), QS-21 (8 μg/mL) 2.83 fold ($p < 0.001$), QS-21 (16 μg/mL) 2.40 fold ($p < 0.01$), QS-21 (32 μg/mL) 2.15 fold ($p < 0.01$), AST VII (2 μg/mL) 1.21 fold ($p < 0.05$), AST VII (4 μg/mL) 1.56 fold ($p < 0.01$), AST VII (8 μg/mL) 1.29 fold, AST VII (16 μg/mL) 1.33 fold ($p < 0.01$), AST VII (32 μg/mL) 2.16 fold ($p < 0.001$), DC-AST VII (2 μg/mL) 2.7 fold ($p < 0.001$), DC-AST VII (4 μg/mL) 2.16 fold ($p < 0.001$), DC-AST VII (8 μg/mL) 2.47 fold ($p < 0.01$), DC-AST VII (16 μg/mL) 2.53 fold ($p < 0.001$), DC-AST VII (32 μg/mL) 3.82 fold ($p < 0.001$), DAC-AST VII (2 μg/mL) 4.19 fold ($p < 0.001$), DAC-AST VII (4 μg/mL) 2.29 fold ($p < 0.01$), DAC-AST VII (8 μg/mL) 3.41 fold ($p < 0.01$), DAC-AST VII (16 μg/mL) 3.67 fold ($p < 0.01$), DAC-AST VII (32 μg/mL) 3.50 fold ($p < 0.001$) compared to PMA- Ionomycin.

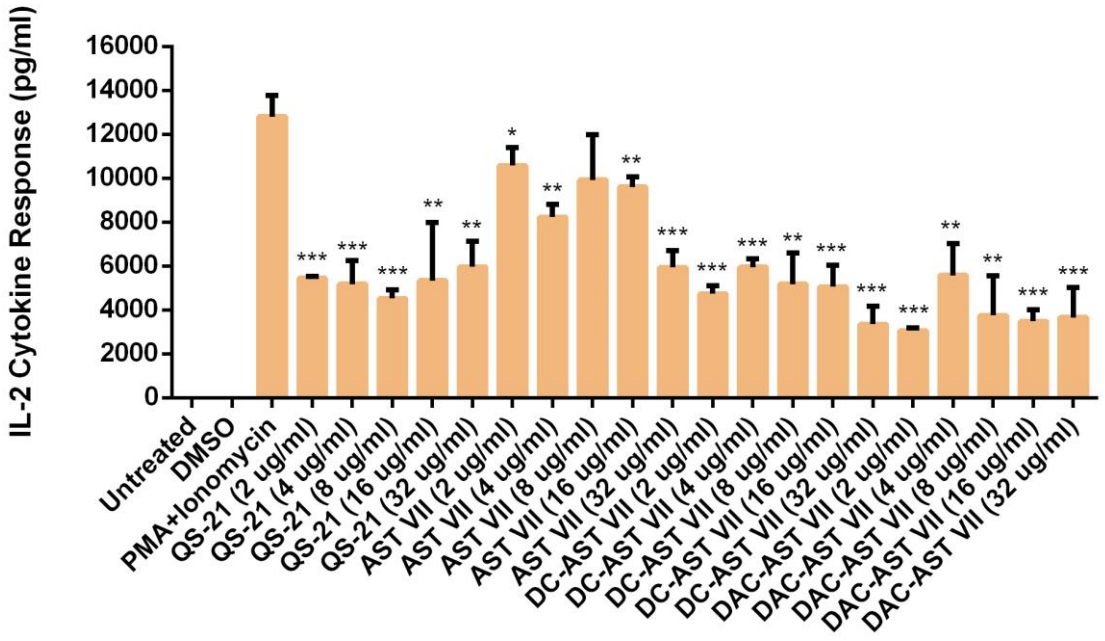


Figure 3.6. Experiment set 2: IL-2 cytokine concentration (pg/mL) in human whole blood stimulated with PMA/ionomycin alone or PMA/ionomycin + different saponin molecules. All groups were performed in triplicate. The significant difference is defined between sample groups and PMA/ionomycin: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

IFN-γ is an important Th1 cytokine in innate and adaptive immune responses for intracellular bacterial and viral infections. IFN-γ promotes macrophage activation, pro-inflammatory cytokine production, differentiation of naive $CD4^+$ T cells to effector Th1 cells, recruitment of lymphocytes. Main sources of IFN-γ are NK, NKT, $CD4^+$ T and $CD8^+$ T cells.[117] As shown in Figure 3.11., following PMA-ionomycin stimulation, all saponin compounds diminished the levels of IFN-γ: QS-21 (2 μg/mL) 2.55 fold ($p < 0.001$), QS-21 (4 μg/mL) 3.05 fold ($p < 0.001$), QS-21 (8 μg/mL) 3.11 fold ($p < 0.001$), QS-21 (16 μg/mL) 3.34 fold ($p < 0.001$), QS-21 (32 μg/mL) 3.07 fold ($p < 0.001$), AST VII (2 μg/mL) 1.22 fold ($p < 0.05$), AST VII (4 μg/mL) 1.33 fold ($p < 0.01$), AST VII (8 μg/mL) 1.14 fold, AST VII (16 μg/mL) 1.10 fold, AST VII (32 μg/mL) 1.25 fold ($p < 0.05$), DC-AST VII (2 μg/mL) 1.51 fold ($p < 0.01$), DC-AST VII (4 μg/mL) 1.36 fold ($p < 0.01$), DC-AST VII (8 μg/mL) 1.51 fold ($p < 0.01$), DC-AST VII (16 μg/mL) 1.44 fold ($p < 0.01$), DC-AST VII (32 μg/mL) 1.63 fold ($p < 0.01$), DAC-AST VII (2 μg/mL) 1.31 fold ($p < 0.05$), DAC-AST VII (4 μg/mL) 1.26 fold, DAC-AST VII (8 μg/mL) 1.22 fold, DAC-AST VII (16 μg/mL) 1.18 fold, DAC-AST VII (32 μg/mL) 1.36 fold ($p < 0.01$) compared to PMA-Ionomycin.

Pro-inflammatory cytokine**,** tumor necrosis factor (TNF)-α have diverse cell functions such as cell proliferation, differentiation, survival and apoptosis. This cytokine is mainly produced by macrophages.[114] As shown in Figure 3.8., all test compounds reduced TNF-α production: QS-21 (2 μg/mL) 2.97 fold ($p < 0.01$), QS-21 (4 μg/mL) 2.52 fold ($p < 0.001$), QS-21 (8 μg/mL) 4.60 fold ($p < 0.01$), QS-21 (16 μg/mL) 2.34 fold ($p < 0.05$), QS-21 (32 μg/mL) 2.63 fold ($p < 0.01$), AST VII (2 μg/mL) 2.15 fold ($p < 0.001$), AST VII (4 μg/mL) 1.94 fold ($p < 0.05$), AST VII (8 μg/mL) 1.75 fold ($p < 0.01$), AST VII (16 μg/mL) 1.75 fold ($p < 0.001$), AST VII (32 μg/mL) 4.21 fold ($p < 0.001$), DC-AST VII (2 μg/mL) 1.95 fold ($p < 0.05$), DC-AST VII (4 μg/mL) 1.92 fold ($p < 0.01$), DC-AST VII (8 μg/mL) 1.75 fold, DC-AST VII (16 μg/mL) 2.04 fold ($p < 0.001$), DC-AST VII (32 μg/mL) 8.46 fold ($p < 0.001$), DAC-AST VII (2 μg/mL) 1.55 fold ($p < 0.05$), DAC-AST VII (4 μg/mL) 1.96 fold ($p < 0.01$), DAC-AST VII (8 μg/mL) 2.09 fold, DAC-AST VII (16 μg/mL) 1.61 fold ($p < 0.01$), DAC-AST VII (32 μg/mL) 2.75 fold ($p < 0.001$) compared to PMA-Ionomycin. Structural difference was not significant in regards to release of TNF-α, the highest doses of AST compounds (32 μg/mL) caused significant decrease in TNF-α, inferring a cytotoxicity issue. Moreover, *Astragalus propinquus* extract was studied on the spleen

of autoimmune depressed mice and showed increase in the production of IL-2 and IL-6 and decrease in TNF-α levels.[121]

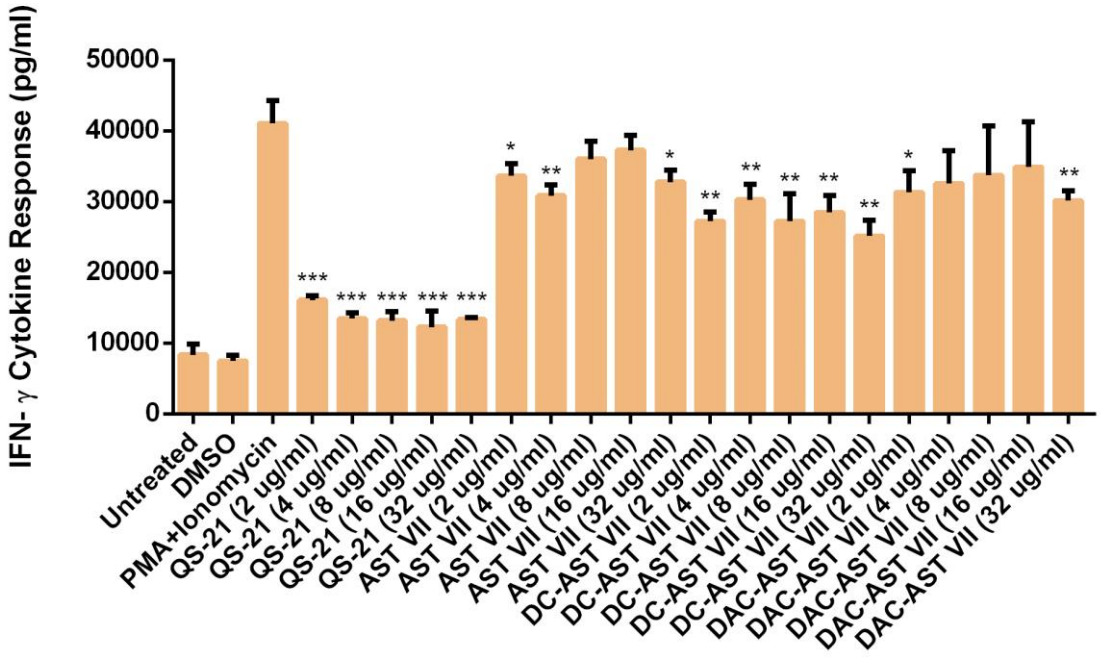


Figure 3.7. Experiment set 2: IFN-γ cytokine concentration (pg/mL) in human whole blood stimulated with PMA/ionomycin alone or PMA/ionomycin + different saponin molecules. All groups were performed in triplicate. The significant difference is defined between sample groups and PMA/ionomycin: *p < 0.05, **p < 0.01, ***p < 0.001

IL-4 is Th2 cytokine, mainly produced by activated T cells, mast cells, basophils and eosinophils. It regulates cell proliferation, apoptosis and expression of numerous genes in lymphocytes, macrophages, fibroblasts, epithelial and endothelial cells.[114] In this study, IL-4 was not stimulated neither with AST VII nor its analog treatments.

The data obtained from cytokine release gave contrary results in comparison to literature. As shown in Table 3.3., AST VII and some of the immunologically active saponin compounds mainly produce IL-1β, IFN-γ and IL-2 cytokines. In our experiments, AST VII, DAC-AST VII, DC-AST VII were found to boost IL-1β production in a dose-dependent manner, while the secretion of IL-2, IFN-γ and TNF-α were suppressed by all compounds. Huang et al., revealed that Ginsenoside Rg1, triterpenoid saponin, induced the secretion of pro-inflammatory cytokines IL-1β, TNF-

α and IL-6 in dose dependent manner in human PBMC derived dendritic cells.[122] Although some triterpenoid saponins, *viz.* Ginsenoside Rg1 and Ginsenoside Rd, were shown to induce Th2 cytokines, AST VII and its derivatives did not stimulate IL-4 production in this study, parallel with previous reports.[55,56] Therefore, we conclude that AST VII and its derivatives do not lead to a Th2 immune response. Zhu et al., shown that Saikosaponin A, which is major triterpenoid saponin isolated from *Radix bupleuri*, inhibited the secretion and mRNA expressions of IL-1β, TNF-α, IL-6 but upregulated IL-10 mRNA expression in LPS induced RAW 246.7 cells.[123] Aslanipour et al., isolated tridesmosidic cycloartane type triterpene glycosides and demonstrated the secretion of IL-2 and IFN-γ.[124]

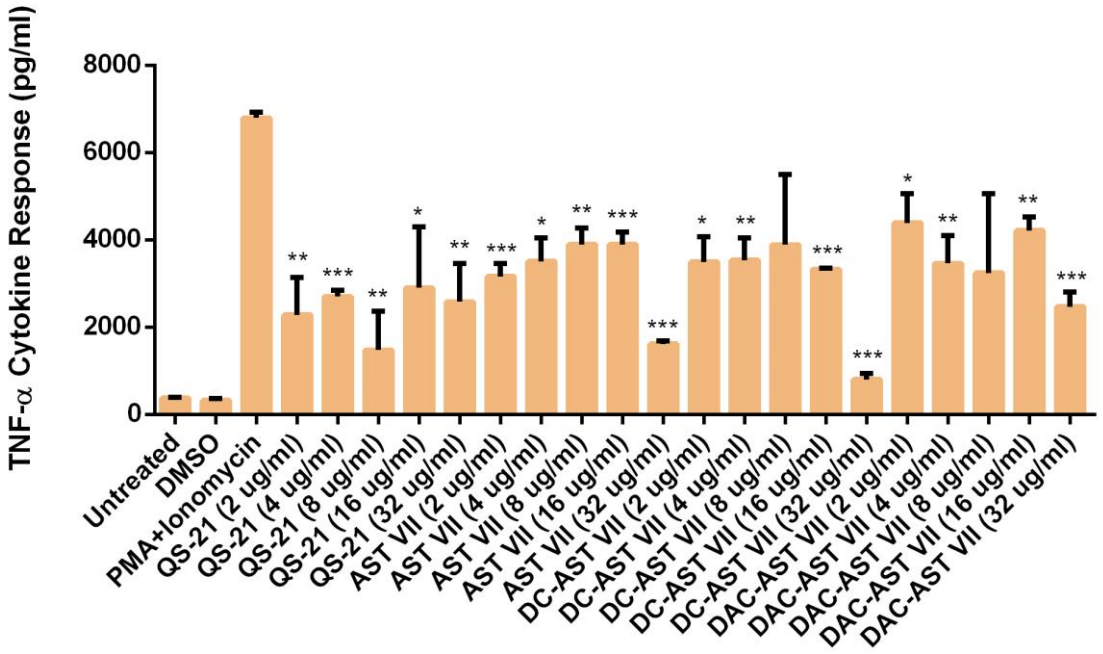


Figure 3.8. Experiment set 2: TNF-α cytokine concentration (pg/mL) in human whole blood stimulated with PMA/ionomycin alone or PMA/ionomycin + different saponin molecules. All groups were performed in triplicate. The significant difference is defined between sample groups and PMA/ionomycin: *p < 0.05, **p < 0.01, ***p < 0.001

Yeşilada et al., studied the cytokine release on human whole blood by treating the cells with *Astragalus* derived saponins. All cycloartane-type saponins elicited IL-1β, IL-8 inducing and TNF-α inhibiting activity in various degree. AST VII was revealed

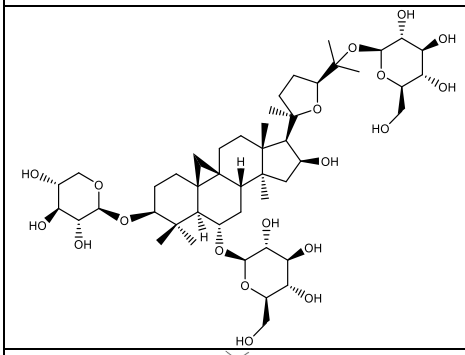
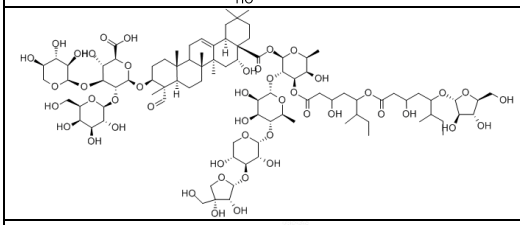
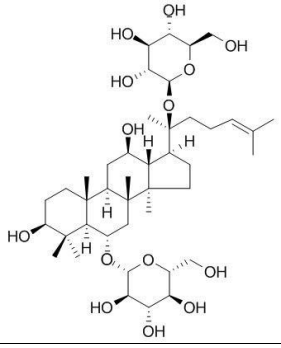
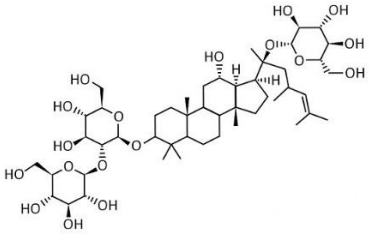
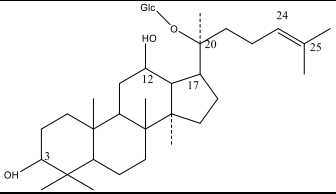
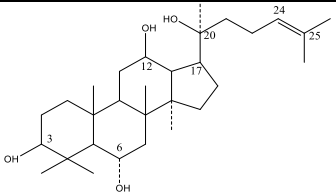
potent IL-2 inducing effect (139.6%), nil effect on IFN-γ, TNF-α (14.7%) inhibitory activity with low IL-1β (36.5%) and IL-8 (34.5%) levels.[125] IL-2 levels for AST VII in our experiment was higher than QS-21 and other AST VII derivatives. However, these levels were also lower compared to PMA-ionomycin stimulated group and were not consistent with data of Yeşilada et al. Nalbantsoy et al. demonstrated that the injection of AST VII alone to Swiss albino mice did not change the cytokine levels in the sera. However, the intraperitoneal injection of LPS (12.5 μg) and AST VII (60 μg) to mice increased the production of IFN-γ, IL-2 and TGF-β, but IL-1β, TNF-α and IL-4 cytokines.[56]

Vitoriano-Souza et al. demonstrated that mouse skin was sensitized with saponin adjuvant (SAP) and cytokine pattern was evaluated at 12, 48 and 168 hours. First 12 h, TNF-α, IL-6, IL-2, IL-17, IL-4 cytokine expressions were induced. TNF-α, IFN-γ, IL-2 cytokines were increased but IL-6, IL-17 and IL-4 cytokines titers were decreased at 48 h. At 168 h, all cytokine levels were diminished till the baseline value.[127]

Coccia et al.,investigated the gene expression profile in draining lymph nodes of C57BL/6 induced by AS01 adjuvant. The data was shown that after the administration of AS01, the gene expression of Th17 related cytokines and interferon, MIF (macrophage migration inhibitory factor), IL-2 related cytokines were increased at 2 hours and 4-6 hours, respectively. MPL and QS-21 synergistic effect was evaluated in terms of higher levels of IFN-γ mRNA expression than MPL or QS-21 alone at 4 hours.[130]

Balance between Th1/Th2 and Th17 cytokines in the microenvironment is crucial because cytokines secreted from a cell type could suppress differentiation of the other cells and production of specific cytokines. For example, IFN-γ, one of the Th1 cytokines, could suppress the naive T cell differentiation to Th2 cells and production of IL-4. The suppressed IL-2, IFN-γ levels following the treatment of AST VII and its analogs might be due to balance phases of Th1/Th2 and Th17 cytokines. Another important issue should also be taken into consideration that the cytokine expression kinetics are subject to alteration depending on the stimulating agent (LPS, PHA, PMA-ionomycin), treated cells and incubation time. In this study, 48 h incubation time probably was not long enough to observe cytokine responses. Therefore, performing time course studies are required before reaching to a conclusion based on our conflicting results in comparison to the previous reports.

Table 3.3. Cytokine release profiles of saponin based compounds isolated from diverse type of plants

Structure	Saponins	Herbs	Activity	References
	Astragaloside VII	*Astragalus trojanus*	IL-2, IL-1β, TNF-α, IL-8	9,125
	QS-21	*Quillaja saponaria*	IFN-γ, IL-2, IL-1β, IL-6, IL-4	26
	Ginsenosides Rg1	Ginseng	Promote IFN-γ, IL-5, IL-6, IL-1β, IL-8	27
	Ginsenoside Rd	*Panax notoginseng*	IFN-γ, TNF-α, IL-2, IL-4, IL-5	28
	M1 Protopanaxadiols		Activation of hmDC maturation, IL-12p70, IL-6 production	29
	M4 20(s)-Protopanaxatriol		Activation of hmDC maturation IL-12p70, IL-6, IFN-γ production	29

3.3.2. IL-1β Secretion Following Treatment of AST VII and its Derivatives on BMDCs and BMDMs

Based on cytokine release studies on WB, induction of IL-1β production following AST VII, DC-AST VII and DAC-AST VII treatments directed our experiments to investigate IL-1β secretion capacity of these saponins on dendritic cells and macrophages. IL-1β production and secretion develop in three steps: a) priming: production of pro-IL-1β; b) processing of pro-IL-1β to mature IL-1β c) release of IL-1β from the cells.[131] Priming step requires stimulation of the TLR/ NF-κB pathway; therefore, lipopolysaccharide (LPS) (TLR4 agonist) is used to activate pro-IL-1β in BMDCs and BMDMs.[132] LPS are major components of the outer membrane of gram-negative bacteria and highly potent activators of the innate immune response. The interaction of LPS with macrophages, granulocytes or dendritic cells via CD14, leads to the synthesis of inflammatory mediators.[133]

BMDCs and BMDMs were treated with LPS (10 ng/mL) and saponins (0.5 μM, 2.5 μM and 10 μM) at the same time and incubated for 6 hours at 37°C in 5% CO_2 incubator. Supernatants were taken to analyze IL-1β concentration by ELISA. As shown in Figure 3.9. and Figure 3.10., LPS increased IL-1β levels. Saponin treatments with LPS substantially boosted IL-1β productions than LPS alone (10 ng/mL). AST VII treatment (922.7 ± 89 pg/mL at 0.5 μM) stimulated more IL-1β titers than DAC-AST VII (686.4 ± 110.2 pg/mL at 0.5 μM) and DC-AST VII (599.9 ± 59.6 pg/mL at 0.5 μM) in BMDCs. AST VII and DC-AST VII have shown similar IL-1β responses in BMDMs. However, IL-1β response was not observed at all following DAC-AST VII treatments probably because of its toxicity towards macrophages. DAC-AST VII was more effective than AST VII in human WB assay but when a specific type of immune cell was investigated, BMDCs produced higher levels of IL-1β after AST VII stimulation. In human WB, neutrophils and monocytes were also capable of producing IL-1β. DAC-AST VII may stimulate these types of immune cells in mixed populations as well as dendritic cells and generate a higher IL-1β titers compared to AST VII and DC-AST VII.

Astragaloside IV (AST IV), which is the cycloartane type triterpene saponin containing xylose and glucose sugar moieties at C-3 and C-6 on the aglycone backbone,

has anti-inflammatory response. Li et al., demonstrated that AST IV inhibited release of the pro-inflammatory cytokines (TNF-α and IL-1β) and the expressions of TLR-4/NF-κB and in LPS induced epithelial cells *in vitro*.[134] Moreover, treatment of AST IV alone did not alter the gene expressions of pro-inflammatory cytokines and adhesion molecules. However, pre-treatment of AST IV inhibited the LPS induced mRNA levels of VACM-1 (Vascular cell adhesion protein 1) (73%), ICAM-1 (Intercellular Adhesion Molecule 1) (60%), E-selectin (79%), P-selectin (71%), MCP-1 (Monocyte Chemoattractant Protein-1) (85%), TNF-α (42%), IL-6 (83%) and TLR4 (30%) in vivo.[135]

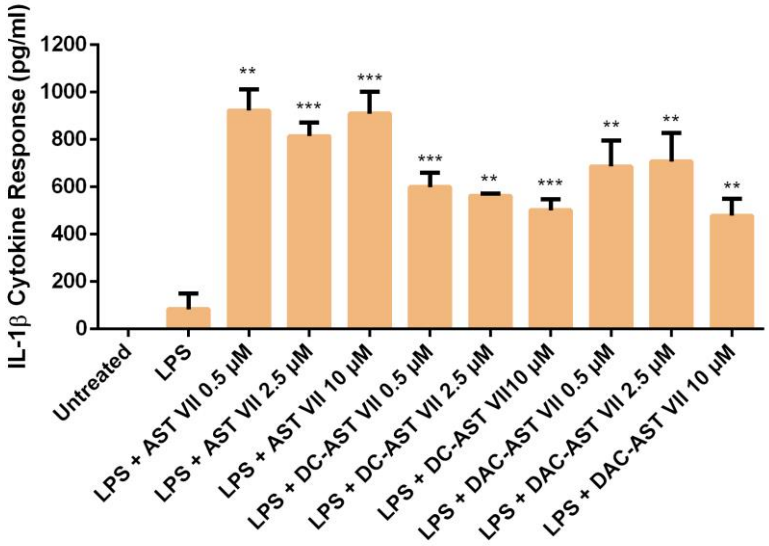


Figure 3.9. IL-1β cytokine concentration (pg/mL) in BMDCs stimulation with LPS and different molecules. All groups were performed in triplicate. The significant difference was calculated compared to LPS alone *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Roix et al. reported that QS-21 was not able to increase the pro-inflammatory cytokines such as IL-6 and IL-1β in mouse BMDCs and BMDMs. However, QS-21 (2 µg/mL) in the presence of TLR4 agonist MPLA enhanced caspase-1/11 and NLPR3 dependent IL-1β production. QS-21 induced IL-1β titers were higher than alum, a very well-known inflammasome activator. In the presence of LPS, AST VII, DC-AST VII, DAC-AST VII induced higher levels of IL-1β in parallel with the mentioned study, but QS-21 did not promote IL-1β secretion in our experiments (data not shown in here). As

we discussed above for DAC-AST VII/IL-1β/BMDM data, higher cytotoxicity of QS-21 might be the reason for the negative results. Moreover, Roix et al. investigated whether inflammasome activation was a general phenomenon for saponins or not. Macrophages were treated with different saponins such as QS-21, digoxin, sapindoside A, hedaracoside C and β-escin in combination with MPLA. Only QS-21 elicited IL-1β response indicating that inflammasome activation may be specific action of *Quillaja* saponins. To prove that hypothesis, *Quillaja* saponin (Quil-A®) and VET-SAP® were administrated to BMDCs/ BMDMs, both of which showed NLRP-3 dependent IL-1β secretion.[102]

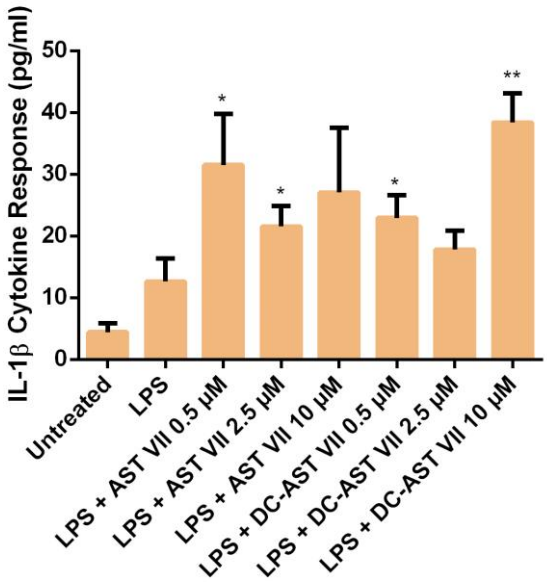


Figure 3.10. IL-1β cytokine concentration (pg/mL) in BMDMs stimulation with LPS and different molecules. All groups were performed in triplicate. The significant difference was calculated compared to LPS alone: *$p < 0.05$, **$p < 0.01$.

LPS induces the gene transcription and translation of IL-1β and further accumulation of pro-IL-1β inside the cells. Incapacity of LPS to recognize NLRs limits the release of IL-1β. Therefore, a second signal is necessary to active caspase-1, which is cleaved the pro-IL-1β to active form, and release IL-1β. As shown in Figure 3.9 and 3.10., LPS alone did not enhanced the secretion of IL-1β as effective as AST VII and its analogs. Synergy between LPS and AST VII boosted the secretion of IL-1β in BMDCs

and BMDMs. As mentioned earlier, the synergistic effect of TLR/NLR to process and release IL-1β leads to think about the activation of NLRs and formation of inflammasomes by AST VII and its analogs.

In the cell plasma membrane, types of lipid and their distributions are not homogenous. Asymmetry between outer and inner leaflets of the membrane is maintained with enzymes such as flippases, scramblases and translocases. Loss of the asymmetry can be associated to apoptosis or presence of pathological condition.[136]

Particulate adjuvants such as alum, poly(lactide-co-glycolide), polystyrene microparticles activate NLRP3 inflammasome and subsequently IL-1β production by dendritic cells. Syk (spleen tyrosine kinase) has a role in the innate cell activation by particulate adjuvants. MSU (monosodium urate) crystals directly engage cell membrane lipids of dendritic cell and trigger the activation and recruitment of Syk by lipid raft aggregation. Syk activates PI3-kinase (phosphoinositol-3-kinase) and further cytokine secretion and phagocytosis. Flach et al. reported that alum does not enter DCs directly and rather delivers the antigen via abortive phagocytosis. Alum interacts with membrane lipids via lipid sorting, recruit ITAM containing molecules Syk and PI3 activation. After these events, dendritic cell uptakes antigen adsorbed on alum, activated, and maturated by expressing MHC II and co-stimulatory molecules.[90]

Welsby et al. stated that Syk relocalization or phosphorylation at lysosomal membrane were not observed in their experiments. However, Syk has a role in promoting cathepsin B proteolytic activity.[108] Moreover, Syk was phosphorylated in monocyte derived DC (moDC) following treatment of QS-21. Syk can be activated with plasma membrane alteration so that it could be changed into lysosomal membrane organization and autophosphorylate.[90,137]

Saponins interact with cholesterol in the cell membrane and involve in membrane permeabilization.[138] AST VII and its analogs may be engaged or interact with dendritic cell membrane and change the lipid organization. Alteration in plasma membrane may transduce a signal for dendritic cell activation. In this process, Syk kinases may have a role in signaling cascade and activate inflammasome to further secrete IL-1β.

To evaluate the structure-function relationship on the activation of NLPR3 inflammasome, chitosan nanoparticles (CNPs), lipid based cubosomes, incomplete Freund's adjuvant and alum were administrated to BMDCs and human peripheral blood

mononuclear cells. Only positively charged CNPs and alum enhanced IL-1β production and inflammasome activation. Li et al. stated that cationic nanotubes induced higher IL-1β compared to anionic and neutral form. Positively charged nanotubes might enhance lysosomal rupture and consequent cathepsin B release.[139,140] IL-1β propagating activity of DAC-AST VII compared to DC-AST VII and AST VII needs to be assessed together with possible hydrolysis of the amide bonds yielding dodecylamine, a positively chargeable residue. Thus, a further experiment to investigate the effect of dodecylamine alone is warranted.

3.3.3 Effects of AST VII and its Derivatives on BMDCs Maturation and Activation

Dendritic cells are potent antigen presenting cells and possess unique capacity to stimulate naive T cell activation and differentiation against a specific antigen. After immature dendritic cell internalize the antigen, they lose the phagocytic capacity and upregulate MHC II and co-stimulatory molecules such as CD86, CD80 to further active naive T cells in lymph nodes. To investigate the effects of AST VII and its derivatives on dendritic cell maturation, bone marrow cells were isolated from B6 mice and differentiated to BMDCs in 10 days by means of stimulation with GM-CSF *in vitro.* GM-CSF induced BMDCs are shown heterogenous population of $CD11c^+$ MHC II^+ cells that comprises of conventional dendritic cells and monocyte-derived macrophages. Both cell populations could undergo maturation but respond differently. Helft et al. searched the marker expressions of $CD11c^+$ MHC II^{high} mature BMDCs and $CD11c^+$ MHC II^{int} immature BMDCs. $CD11c^+$ MHC II^{high} mature BMDCs expressed surface markers restricted to dendritic cells (CD135, CCR7, CD117) whereas $CD11c^+$ MHC II^{int} immature BMDCs upregulated markers related to macrophages and monocytes (CD64, CD115, CD14)[141]. Therefore, BMDCs gating were done in two subpopulations as $CD11c^+$ MHC II^{high} mature BMDCs and $CD11c^+$ MHC II^{int} immature BMDCs as shown in Figure 3.11.

BMDCs ($2x10^5$ cell/well) were cultured in 96 well tissue plate and stimulated with AST VII for 24 hours at concentrations of 1, 3, 6, 11 μM and 11, 16, 21, 32, 64 μM in two different experiment sets. TLR4 agonist LPS (10 ng/mL) was used as positive control because of its capacity to mature dendritic cells. BMDCs maturation state was

evaluated by analyzing MHC II, CD80 and CD86 expressions by flow cytometry and secretion of IL-12 by ELISA. AST VII alone did not induce the expression of MHC II, CD80 and CD86 markers and IL-12 cytokine production even at higher concentrations as shown in Figure 3.12.

The spleen was collected from B6 mice to investigate if AST VII alone could enhance dendritic cell maturation with the help of other cell types or secreted cytokines in the microenvironment. Single cell suspension of splenocytes were treated with AST VII alone at concentrations of 5, 11, 21 μM. However, AST VII did not stimulate the upregulation of MHC II, CD80 and CD86 maturation markers. Based on BMDCs and splenocyte culture results, it was concluded that AST VII alone did not have the capacity to induce maturation of BMDCs *in vitro*.

These data are consistent with the literature. Sun et al. treated GM-CSF induced BMDCs for 16 hours with OVA and different adjuvants such as LPS, alum, NISV (non-ionic surfactant vesicles were prepared from mono-palmitoyl glycerol, cholesterol, and dicetyl phosphate) and PLGA [poly (d,l-lactic-co-glycolic acid)]. LPS clearly activated the expression of MHC Class II, CD40, CD80, CD86 compared to DPBS (Phosphate Buffer Saline) treated dendritic cells. None of these adjuvants in the study induced MHC Class II, CD40, CD80 and CD86 comprehensively as LPS did. Although at higher doses of PLGA microparticles, some rises in MHC Class II, CD40 and CD86 were observable, which was not higher than background levels observed in the groups treated with soluble OVA in the absence of adjuvants.[96]

Wilson et al. demonstrated that ISCOMATRIX adjuvant failed to increase MHC II, CD80, CD40 expressions compared to LPS and CpG adjuvants as well as producing pro-inflammatory cytokines *in vitro* Flt3 (FMS-like tyrosine kinase 3) ligand or GM-CSF induced dendritic cells. To evaluate activation status of DCs *in vivo*, ISCOMATRIX adjuvant was injected to mice and DCs were isolated from draining lymph node (DLN). *In vivo* results demonstrated that $CD8^+$ $CD205^+$ DCs elicited upregulation of CD40, CD80, CD86, MHC II, and plasmacytoid DCs revealed modest activation. $CD8^-$ $CD205^-$ DCs or tissue derived $CD8^-CD205^+$ DCs, spleen and non-DLN did not show any significant effect following ISCOMATRIX treatment.[142] Moreover, as ISCOMATRIX, Matrix C did not induce upregulation of maturation markers (CD86, CD80) in BMDCs *in vitro.*[143] Saponin based adjuvants including AST VII did not elicit DCs maturation alone *in vitro*. Therefore, we suggest that these group of adjuvants are not able to bind specific receptors for DCs maturation as TLR agonists (LPS, CpG) do.

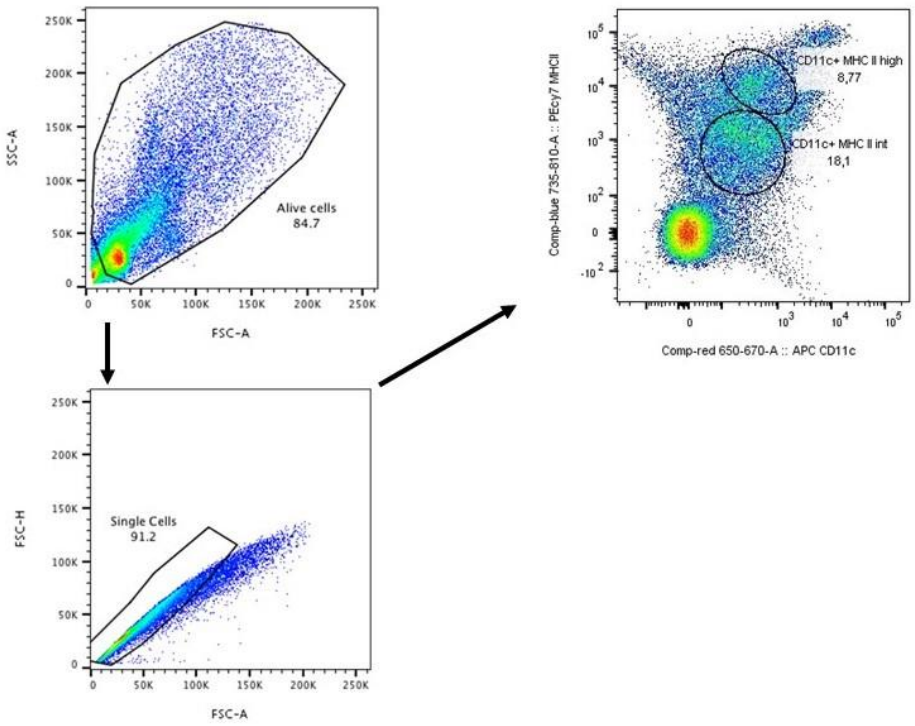


Figure 3.11. BMDC gating strategy. Alive cells were determined with forward scatter area (FSC-A) and side scatter area (SSC-A). Single cells were separated with FSC-A and forward scatter height (FSC-H). Activation state of BMDCs following stimulation was analyzed by gating single cells through CD11c and MHC II expression. $CD11c^{+}$ MHC II^{high} BMDC and $CD11c^{+}$ MHC II^{int} BMDC were used in analysis.

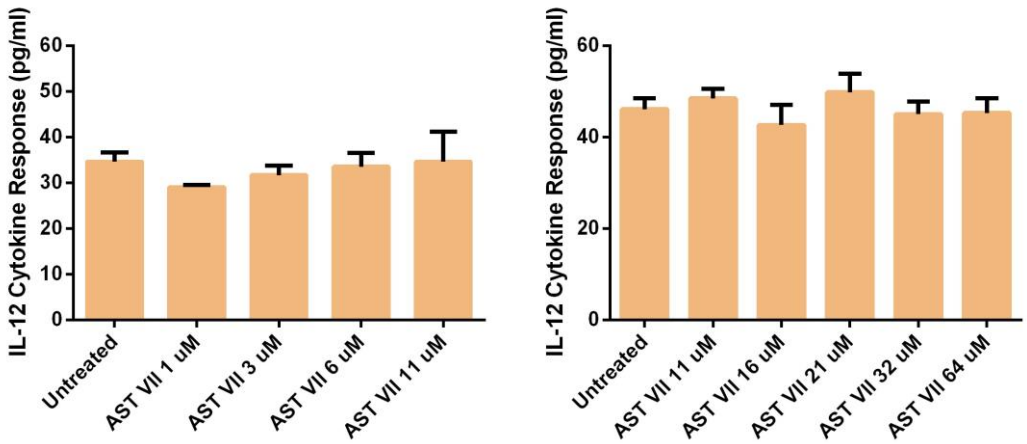


Figure 3.12. IL-12 cytokine response following treatment of AST VII at concentration of 1, 3, 6, 11, 16, 21, 32, 64 μM.

The data obtained from IL-1β production in BMDCs revealed that AST VII was able to increase IL-1β levels significantly in the presence of TLR4 agonist LPS (10 ng/mL). Therefore, BMDCs were treated with LPS 10 ng/mL and AST VII at concentrations of 2, 5, 10 μM at the same time and were incubated for 24 h.

Expressions of MHC II, CD86 and CD80 markers were increased following stimulation of AST VII and LPS. In $CD11c^{+}$ MHC II^{high} BMDCs, MHC II expression was enhanced by treatment of the cells with AST VII 2 μM (11160 MFI), AST VII 5 μM (10533 MFI), AST VII 10 μM (12081 MFI) compared to the untreated group. AST VII 10 μM significantly enhanced MHC II expression compared to LPS (10 ng/mL) ($p < 0.05$) (Figure 3.13.a). Expression of the co-stimulatory molecule CD86 was altered in AST VII 2 μM (370.7 MFI), AST VII 5 μM (327 MFI), AST VII 10 μM (421 MFI). AST VII 2 μM ($p < 0.01$) and AST VII 5 μM ($p < 0.001$) remarkably stimulated CD86 expression compared to LPS (Figure 3.13.b). AST VII 2 μM (145 MFI), AST VII 5 μM (141 MFI), AST VII 10 μM (149 MFI) induced CD80 expression. As CD86, AST VII 2 μM and AST VII 10 μM significantly upregulated CD80 markers compared to LPS ($p < 0.05$) (Figure 3.13.c). The relative percentages of $CD11c^{+}$ MHC II^{high} BMDCs that express CD86 and CD80 after LPS treatment with/out AST VII were illustrated in Figures 3.14. and 3.15. Moreover, AST VII (10 μM)-LPS co-stimulation increased IL-12 secretion compared to LPS alone ($p < 0.01$) (Figure 3.16.)

Takei et al. demonstrated that HLA-DR, CD1a, CD80, CD83, CD86 maturation markers were increased via stimulation of human monocyte derived DC (hmDC) with M1 and M4, derived from the protopanaxadiols and protopanaxatriols. IL-12p70, IL-6 cytokine production was higher than LPS treated hmDC.[129] Huang et al. revealed that Ginsenoside Rg1 induced the expression of HLA-DR, CD83, moderate expression of CD80 and low expression of CD14 in human PBMC.[122]

There are contradictory reports for the immunostimulatory activity of LPS and *Astragalus* extracts. Shao et al. reported that *Astragalus membranaceus* (*Ast. mem*.) polysaccharide (ASP) activated mouse B cells and macrophages but not T cells in terms of production of IL-1β and TNF-α cytokines. LPS levels in ASP were measured as 0.15‰. Therefore, activity of *Ast. mem*. ASP was not due to the presence of LPS.[144] *Astragalus mongholicus* polysaccharides (*Ast. Mong.* ASP) isolated from *Astragalus mongholicus* were investigated for their immunomodulatory effects on murine BMDCs *in vitro*. *Ast. mong.* ASP (10, 50, 100, 250 μg/mL) increased the co-expression of

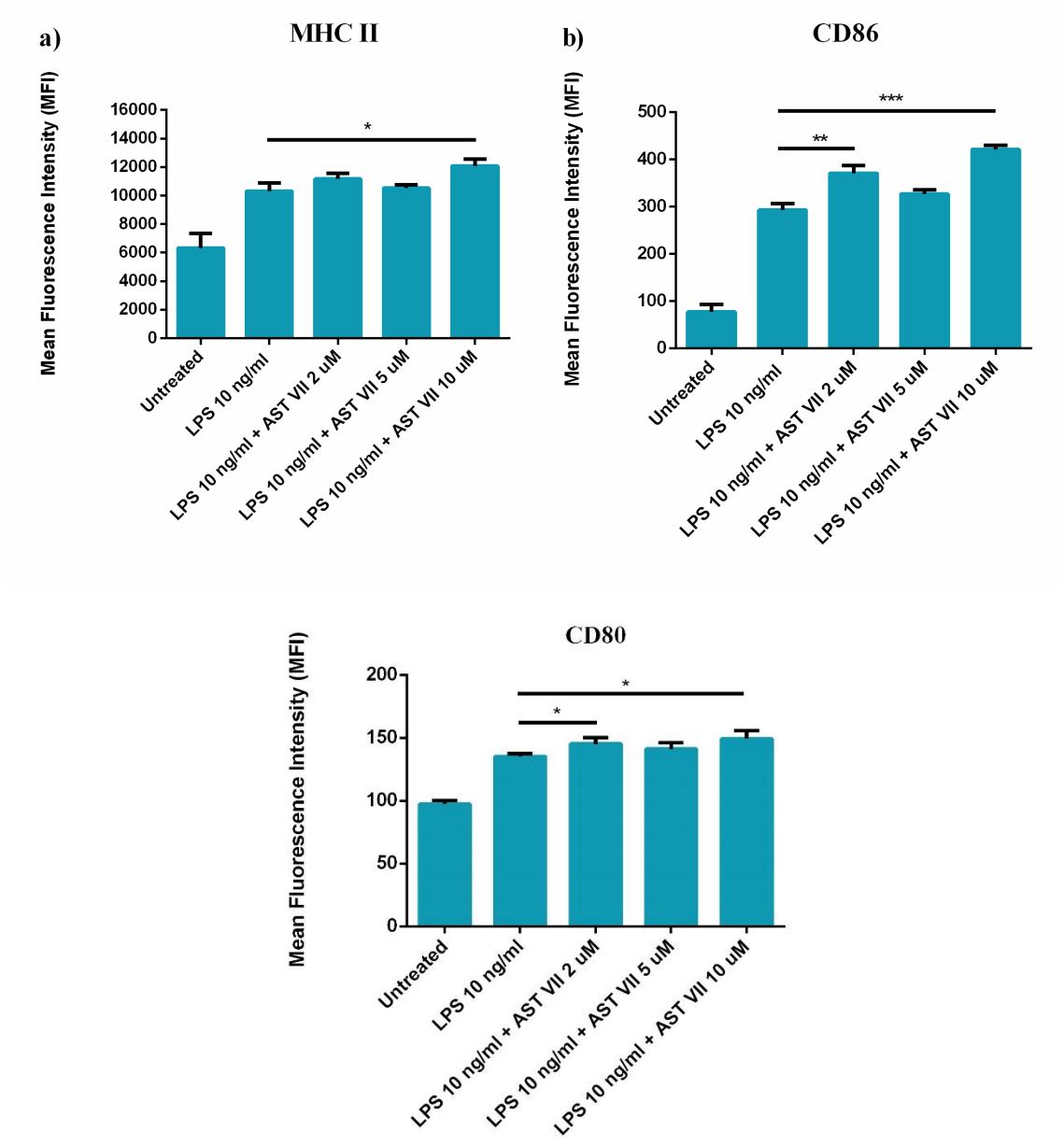


Figure 3.13. Maturation marker profiles of $CD11c^{+}$ MHC II^{high} gated BMDCs treated with LPS 10 ng/mL and AST VII (2, 5, 10 μM). a) MHC II expression b) CD86 expression c) CD80 expression. Significant differences comprised with LPS, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

CD11c and MHC class II molecules on DCs surface, and 100 μg/mL concentration was optimal for treatment.[145] Activity-endotoxin concentration relationship revealed that the activity was not deriving from the contamination of extracts, but it was instead due to lysis of the endophytic bacteria, colonizing the internal tissue of *Astragalus membranaceus* plant material. Therefore, LPS was a common constituent and had a role in immune regulatory activity.[146]

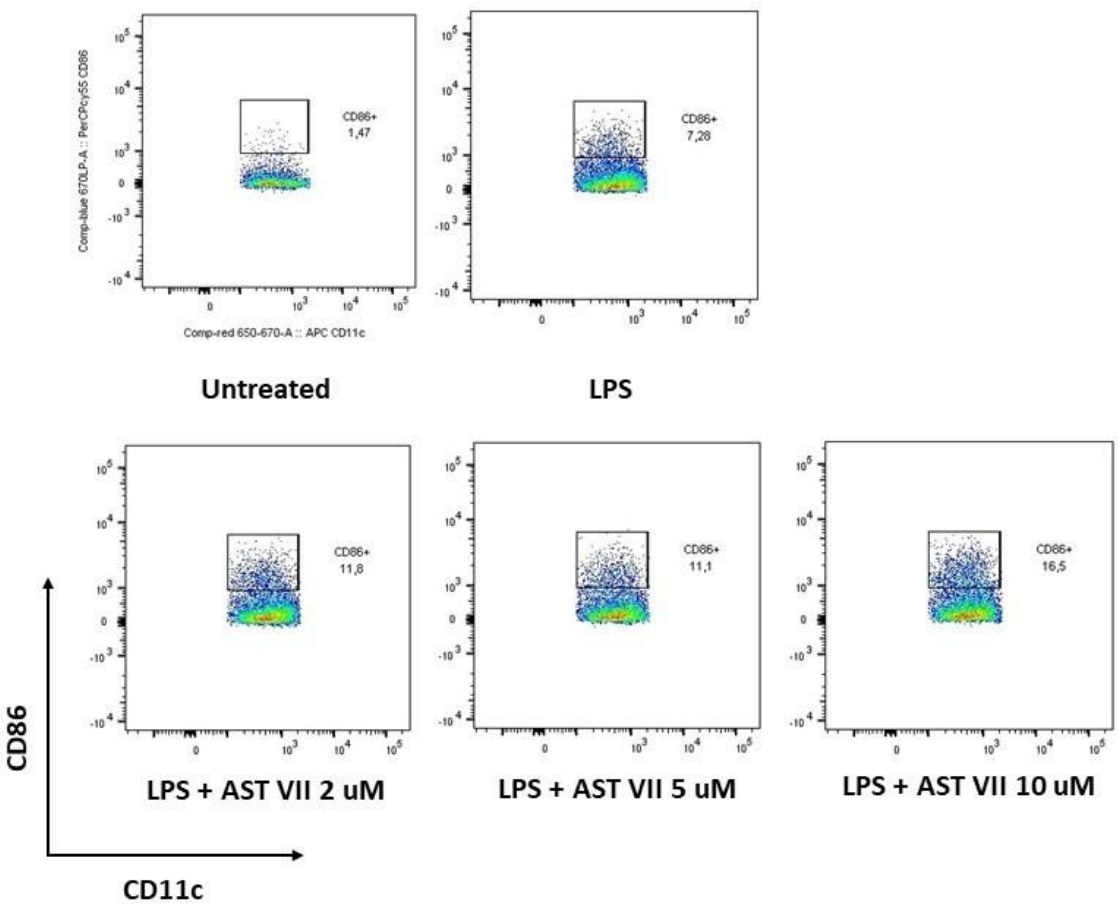


Figure 3.14. The relative percentage of $CD11c^{+}$ MHC II^{high} BMDCs that express CD86 after LPS treatment with/out AST VII.

TLR4 agonist LPS/MPL induced NF-κB pathway to produce pro-inflammatory cytokines. As shown in previous experiments, AST VII did not exhibit NF-κB activation in THP-1 cell line.[147] Therefore, activation of inflammasome, IL-1β production and after that DCs maturation and activation need co-stimulation with TLR4 agonists *in vitro*.

DAC-AST VII and DC-AST VII were subjected to BMDCs for 24 h in the absence/presence of LPS. Without LPS, DC-AST VII and DAC-AST VII did not upregulate MHC II; however, CD86 and CD80 marker expressions were enhanced [CD86 expressions: DC-AST VII (2 μg/mL) 1.17 fold ($p < 0.05$), DC-AST VII (5 μg/mL) 1.31 fold, DC-AST VII (10 μg/mL) 1.31 fold, DAC-AST VII (2 μg/mL) 1.15 fold ($p < 0.05$); For CD80 expressions: DC-AST VII (2 μg/mL) 1.22 fold ($p < 0.05$), DC-AST VII (5 μg/mL) 1.24 fold ($p < 0.05$), DC-AST VII (10 μg/mL) 1.24 fold

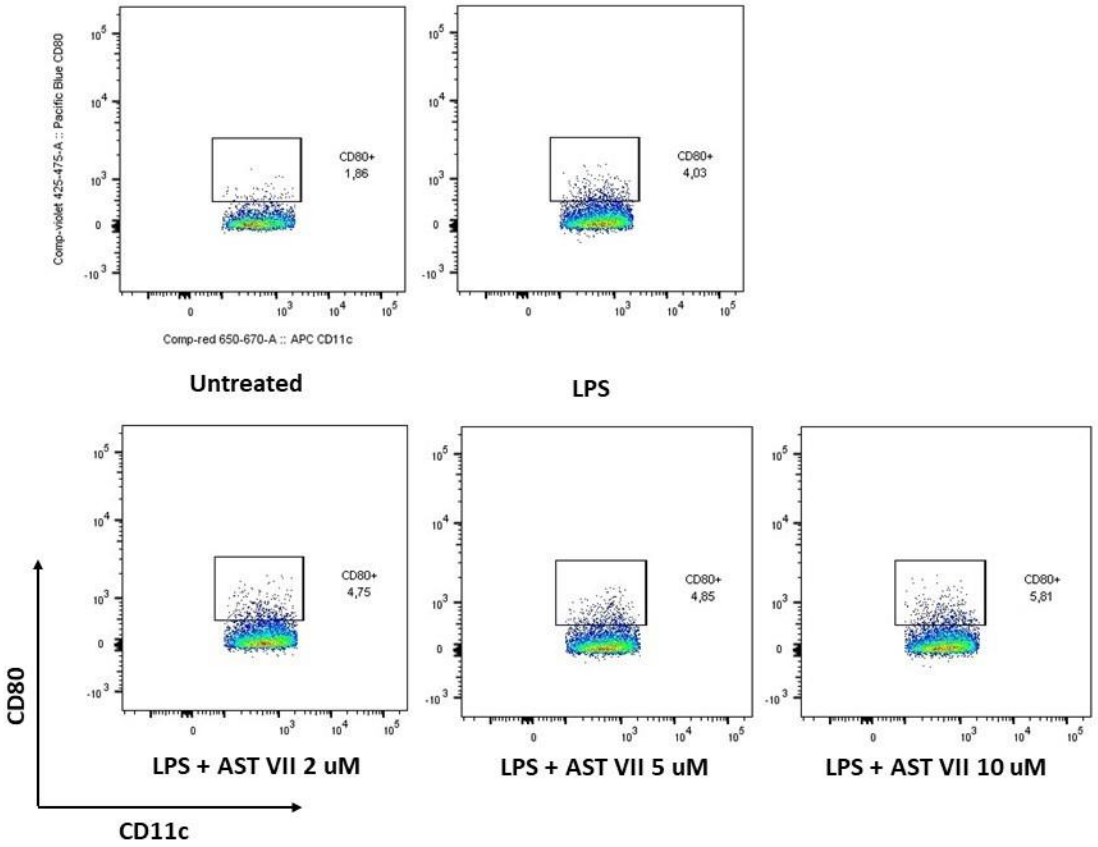


Figure 3.15. The relative percentage of $CD11c^{+}$ MHC II^{high} BMDCs that express CD86 after LPS treatment with/out AST VII.

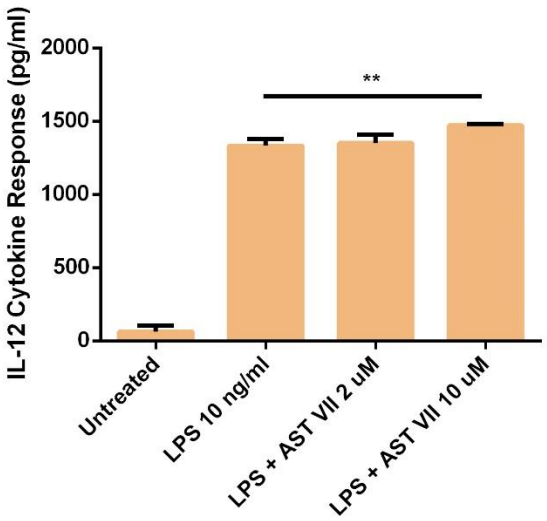


Figure 3.16. IL-12 cytokine response after the treatment of BMDCs with LPS 10 ng/mL and AST VII 2 μM and 10 μM

($p < 0.01$), DAC-AST VII (2 μg/mL) 1.13 fold, DAC-AST VII (5 μg/mL) 1.13 fold ($p < 0.01$), DAC-AST VII (10 μg/mL) 1.14 fold ($p < 0.01$), DAC-AST VII (20 μg/mL) 1.12 fold ($p < 0.01$) compared to untreated group (Figure 3.17.)]. The upregulation of CD86 and CD80 induced by DC-AST VII were higher than DAC-AST VII. Therefore, carboxylic acid moieties on the structure or hydrophilic characteristic of DC-AST VII have an effect on dendritic cell activation.

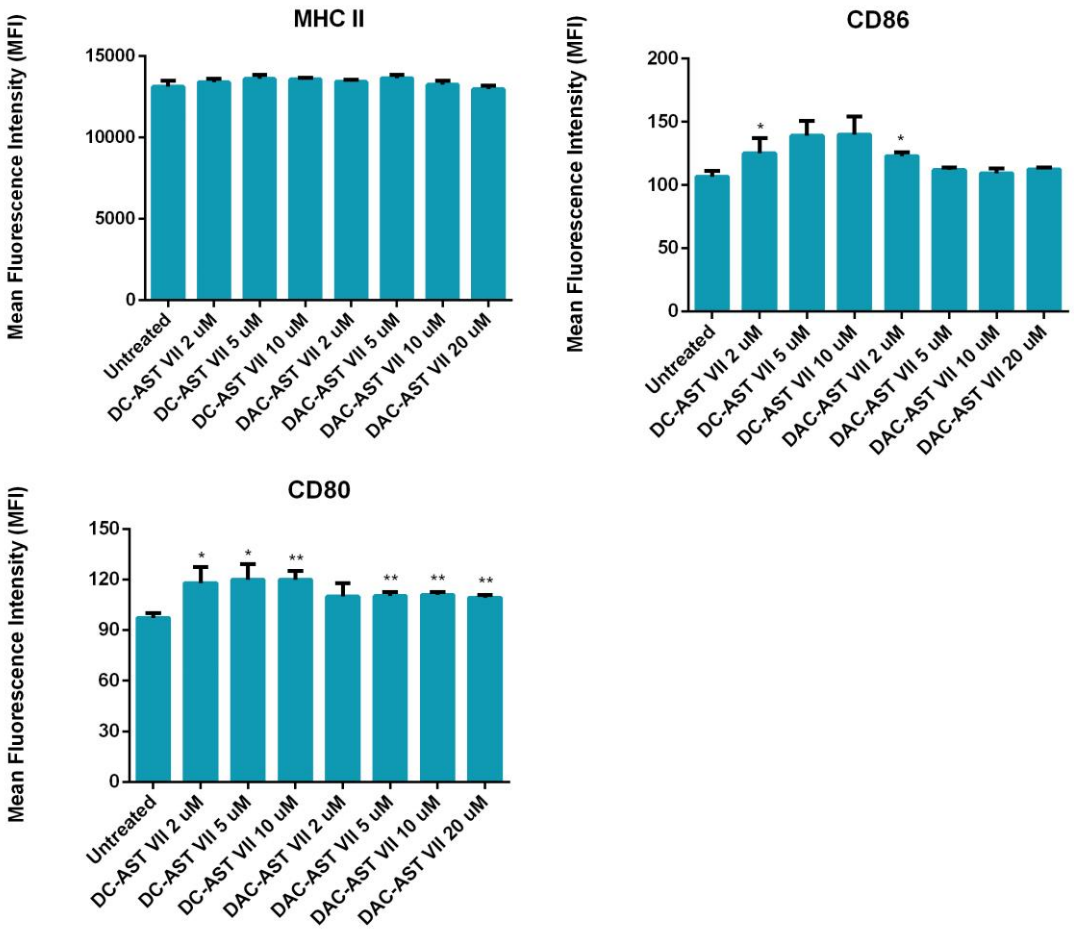


Figure 3.17. MHC II, CD86 and CD 80 Surface Marker Expression Following DC-AST VII and DAC-AST VII treatments. Significant differences stated compared to untreated group, *$p < 0.05$, **$p < 0.01$.

In the presence of LPS, DC-AST VII did not affect the MHC II, CD86 and CD80 expressions. However, DAC-AST VII stimulation with LPS significantly suppressed the expression of MHC II [1.28 fold ($p < 0.001$)], CD86 [1.58 fold ($p < 0.001$)] and CD80 [1.29 fold ($p < 0.01$)]. It can be stated that DC-AST VII and DAC

AST VII did not require LPS to upregulate CD86 and CD80. However, the reason for unchanged MHC II levels on dendritic cell was unclear. As a result, these compounds can activate dendritic cell in a different way compared to AST VII.

3.3.4. Saponin Induced Antigen Presentation and Cross Presentation

Activation status of DCs can be determined by investigating cytokine response in the microenvironment and co-stimulatory molecule expression on the cell surface. These approaches give indirect information about potency of DCs to activate T cells. Analysis of T cell response in a co-culture system is a more direct approach to evaluate T cell activation, proliferation and polarization.[148] For these purposes, co-stimulation independent B3Z and co-stimulation dependent allogenic mixed leukocyte reaction assays were performed.

3.3.4.1. Co-stimulation Independent Cross Presentation by B3Z Assay

Binding of peptide/MHC complexes on the surface of APC with TCR on the T cells result in the generation of intracellular signals in T cells. This initial recognition event in single T cell could be determined by lacZ assay. lacZ assay utilizes T cell hybridomas, which have a bacterial 3-galactosidase gene (lacZ) under the transcriptional control of the nuclear factor of activated T cells (NF-AT) element of interleukin 2 (IL-2) enhancer. Following TCR triggering, T cells begin to secrete IL-2 as well as accumulate lacZ intracellularly. lacZ expression can be visualized by loading the T cells with either fluorogenic or chromogenic substrates.[149]

MHC I presentation of antigen-derived epitopes was determined by using the B3Z (CD8 T cell) hybridoma. B3Z cells are $CD8^+$ T cells that express T cell receptor specific for MHC I and SIINFEKL peptide (OVA 258-265) derived from OVA protein[150]. This assay is co-stimulation independent so that expression of co-stimulatory molecules such as CD80, CD86 is not important for the activation of T cell. Induction of $CD8^+$ T cells response is dependent on the ability of stimulants to facilitate cross-presentation of exogenous antigen on the MHC I molecule by DCs.[83]

Different experiment sets were studied to optimize the B3Z protocol. BMDCs from B6 mice were treated with OVA protein (100 μg/mL) with/out AST VII (5 μM) for 5 hours. After that the cells were treated with LPS (10 ng/mL) for 24 hours to complete DCs maturation process and B3Z cells were added to appropriate wells and incubated for 18 hours. The supernatants were collected and stored at -20ºC for further ELISA assays. Readout of B3Z was made by adding Z buffer to each well and measuring OD value at 595 nm at spectrophotometer.

In another experiment set, BMDCs were firstly treated with LPS (10 ng/mL) and then stimulated with OVA/AST VII (5 μM) and further added B3Z cells. As shown in Figure 3.18. and Figure 3.19., OVA treatment alone or with AST VII did not alter the LacZ expression compared to negative control (treated by medium). Maturation status of BMDCs did not affect the MHC class I presentation of OVA protein with/out AST VII to B3Z cells. In addition, DAC-AST VII and DC-AST VII treatment alone did not promote $CD8^+$ T cell response. To confirm cross presentation activity of AST VII and its derivatives, they could be administrated to mice and after that lymph nodes could be collected to make ex vivo cross presentation tests.[151]

As shown in Figure 3.18. and 3.19., even LPS treatment, BMDCs did not show MHC I cross presentation of OVA whole protein to $CD8^+$ T cells. It could result from *in vitro* produced GM-CSF induced BMDCs, which do not have the ability to cross present the antigen. As mentioned before, GM-CSF induced BMDCs showed heterogenous population of DCs and macrophages. The cross presentation capacity of various DCs subsets has been studied in mouse systems and $CD8\alpha^+$ DCs resident in lymphoid tissues are highly specialized for cross presentation of exogenous antigens.[152]

Den Brok et al., revealed that adjuvants such as MF59, $ALPO_4$, Miglyol were not able to be cross-presented OVA protein on the surface of DCs in B3Z assay. Low amount of OVA protein did not give detectable results as we obtained from our experiments. Moreover, MHC II restricted OVA presentation to OT-II cells (CD4 T cell recognized ovalbumin 323-339) did not change with administration of saponin based adjuvant (SBA). SBA induced cross presentation of OVA is co-stimulation independent. SBA did not change the expression of MHC-I/II, CD206 (mannose receptor), CD80, CD86 levels on the *in vitro* cultured DCs. Maturation of DCs did not have an effect on the increase in B3Z cells cross presentation as we shown in our experiments. Moreover, increases in the cross-presentation activity of DCs induced by SBA are not dependent on antigen uptake, type I IFN receptor or the inflammasome

component NLRP3. To investigate SBA potential *in vivo*, ISCOM and OVA were administrated to mice and after 12 hours post-injection, draining lymph nodes were isolated. In vivo experiments were revealed that lymph node resident and migratory $CD11b^+$ DCs have the capacity to cross-presented OVA following SBA treatment.[83]

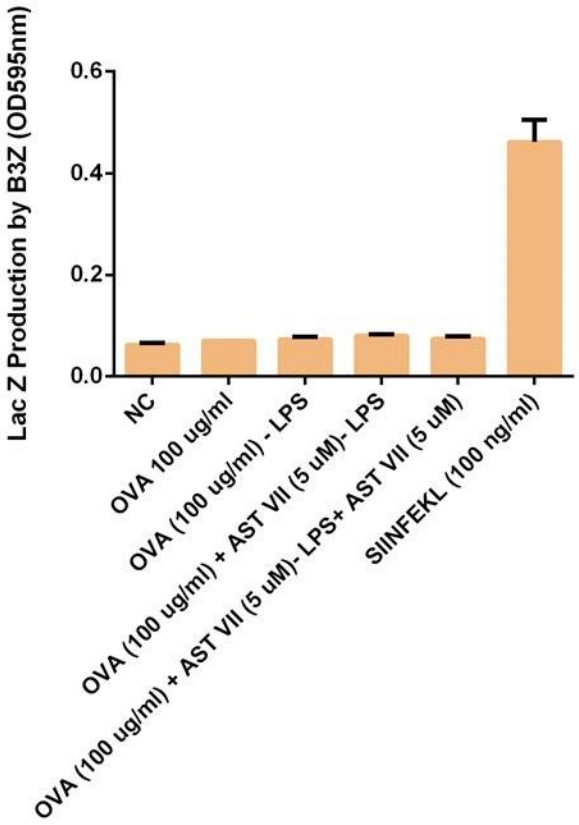


Figure 3.18. LacZ production by B3Z cells at OD595 nm. BMDCs were treated with OVA whole protein 100 μg/mL and OVA + AST VII 5 μM and SIINFEKL as a control for 5 hours. After washing with DPBS, BMDCs were treated with LPS and LPS + AST VII 5 μM for maturation for 24 hours. B3Z cells were added and incubated for 18 hours. B3Z assay readout by spectrophotometry at 595 nm absorbance. NC: negative control (only medium).

Intracellular delivery of the antigen and induction of CTL response have been revealed in nanoparticles systems such as liposomes, PLGA particles etc.[153] Matrix C ISCOMs was able to cross present OVA protein in the GM-CSF induced BMDCs to B3Z cells.[143] Matrix C ISCOMs provide a delivery system for OVA and promote the internalization of the antigen and saponin adjuvant at the same DCs. Moreover, Matrix

C adjuvant was more effective than Quil-A alone to cross-present OVA to B3Z cells.[143] Therefore, a delivery system containing AST VII could be designed to improve its cross-presentation potential.

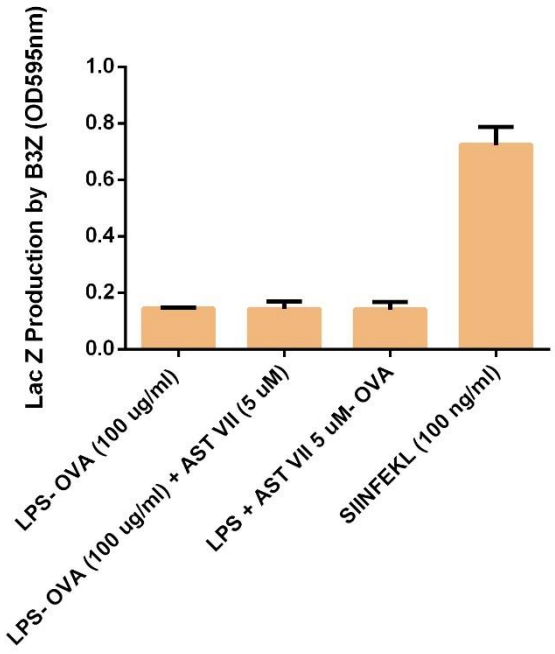


Figure 3.19. LacZ production by B3Z cells at OD595. BMDCs were treated with LPS (10 ng/mL) or LPS and AST VII 5 μM for 24 hours to mature BMDCs. After then, OVA whole protein 100 μg/mL, AST VII and SIINFEKL were added to BMDCs for 5 hours. After washing with DPBS, B3Z cells were added and incubated for 18 hours. B3Z assay readout by spectrophotometry at 595 nm absorbance.

3.3.4.2 Allogenic Mixed Leukocyte Reaction

Mixed leukocyte reaction is a simple and efficient *in vitro* model to investigate T cell activation and proliferation. In MLR, self-peptide presented on MHC molecules on the surface of DC is recognized by T cells, which have never encountered with this antigen in thymic development.[154]

Bone marrow from Balb/c mice was taken and differentiated into BMDCs with GM-CSF. BMDCs were treated with stimulating agents such as LPS, AST VII, DAC-AST VII, DC-AST VII and incubated for 24 hours at 37ºC 5% CO_2 incubator. Next day,

naive $CD4^+$ and $CD8^+$ T cells were isolated from the spleens of B6 mice by negative selection kits and co-cultured with BMDCs in 1:5 ratio (BMDCs: T cells) for 3 days. After incubation, T cells were collected from the culture and prepared for FACS analysis. Cell viability was determined by Ghost dye BV 510 dye. Cell surface marker analysis was made by CD8α- PerCp-Cy5.5, CD4- Pacific Blue, CD44- APC. To gate T cells, lymphocytes were chosen according to FSC-A and SSC-A. Alive cells were gated in SSC-A and Violet 510. In the gated alive cells, CD4 and CD8 markers were placed in XY axis and gated. $CD4^+$ T cells and $CD8^+$ T cells were analyzed to express CD44, T cell activation marker (Figure 3.20).

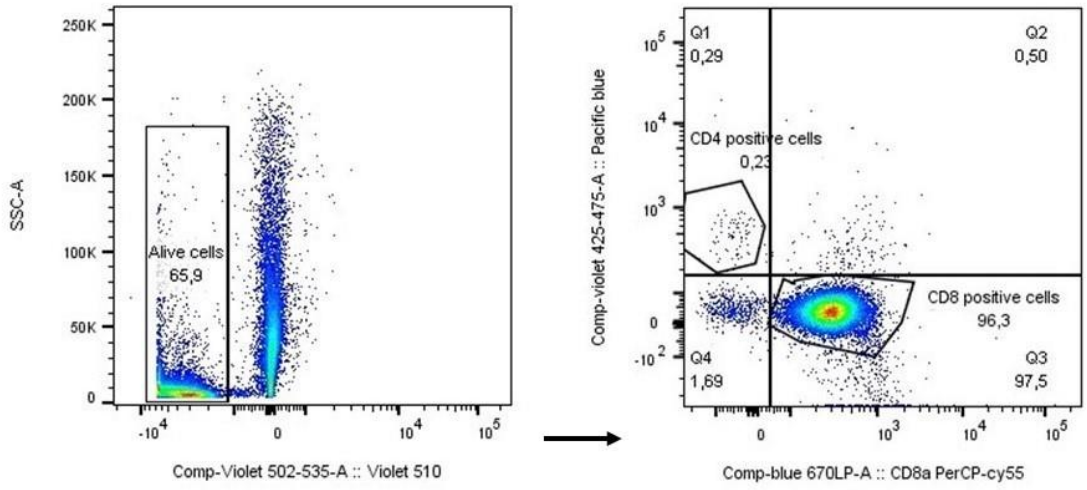


Figure 3.20. Gating strategy for T cells, which are obtained from mixed leukocyte reaction assay. T cells are gated in terms of FSC-A/ SSC-A and then alive cells were chosen by Violet 510 dye. Alive cells were gated by CD8a and CD4 markers. CD8 positive T cells and CD4 positive T cells were analyzed to express CD44 expression marker.

As shown in Figure 3.21., LPS 10 ng/mL (1.28 fold, $p < 0.05$), LPS (10 ng/mL) + AST VII (5 μM) (1.26 fold, $p < 0.05$), AST VII 5 μM (1.29 fold), DA 10 μM (1.49 fold, $p < 0.05$), DC 10 μM (1.26 fold) increased CD44 activation marker in $CD8^+$ T cells compared to negative control (NC-only T cells without stimulation). AST VII alone was able to stimulate $CD8^+$ T cells similar to LPS. LPS + AST VII 5 μM treatment did not alter the $CD8^+$ T cell response compared to LPS and AST VII alone. It was proved that the activation of $CD8^+$ T cells by AST VII was not dependent on LPS stimulation. DAC-AST VII (10 μM) was more effective in the activation of $CD8^+$ T cell

than DC-AST VII (10 μM). At the same concentration, DAC-AST VII (5 μM) was more potent than AST VII (5 μM) in terms of $CD8^+$ T cell activation.

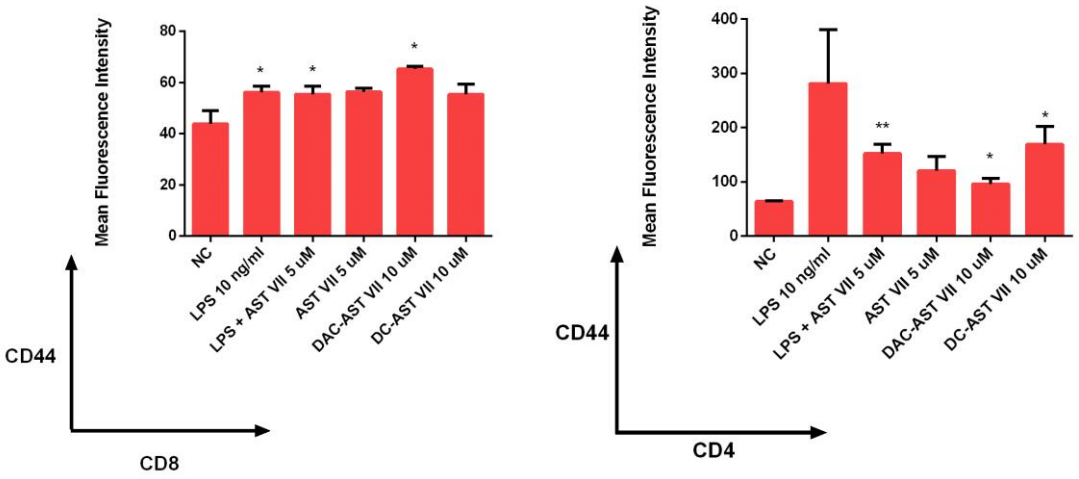


Figure 3.21. CD44 activation marker expression in $CD8^+$ T cells and $CD4^+$ T cells. BMDCs were treated with LPS 10 ng/mL, LPS 10 ng/mL and AST VII 5 μM, AST VII 5 μM, DAC-AST VII 10 μM, DC-AST VII 10 μM and incubated for 24 hours. After the incubation, CD8+ T cells and CD4+ T cells were isolated from B6 mice with negative selection, added to BMDCs and incubated for 3 days. T cells in the culture were collected and stained for CD44 expression. The significant difference is defined compared to samples with NC (negative control). $p^* < 0.05$, $p^{**} < 0.01$

In $CD4^+$ T cells, LPS 10 ng/mL (4.4 fold), LPS (10 ng/mL) + AST VII (5 μM) (2.37 fold, $p < 0.01$), AST VII 5 μM (1.87 fold), DAC-AST VII 10 μM (1.5 fold, $p < 0.05$), DC-AST VII 10 μM (2.6 fold, $p < 0.05$) amplified CD44 expression in $CD4^+$ T cells compared to negative control (NC). Co-stimulation of AST VII with LPS was increased higher CD44 expression in $CD4^+$ T cells than AST VII alone. DC-AST VII (10 μM) was more proficient in CD44 expression on $CD4^+$ T cell than DAC-AST VII (10 μM). CD44 upregulation was shown similar response between DC-AST VII (5 μM) and AST VII (5 μM) treatments.

CD44, a carbohydrate binding transmembrane protein, interacts with the hyaluronan and localizes in lipid rafts close to TCR complex and upregulates after TCR engagement. Once CD44 upregulated in T cells, its expression sustained in effector cells and memory cells till the immune response is weaken. Besides activation and

memory markers, CD44 elicited many biological functions as T cell migration to infection site, activating TCR signaling-associated src family kinases Lck and Fyn, providing resistance of activated T cell to apoptosis, interacting with cytoskeleton and acting like co-receptor in T cell activation.[155,156] Increase in CD44 expression following adjuvant treatments were indicated the activation of $CD4^+$ and $CD8^+$ T cells. As LPS is powerful adjuvant, CD8 T cell activation was shown similar response with administration of AST VII and its analogs compare to LPS.

Increased levels of IL-1β and IL-17A in whole blood and IL-1β on BMDCs following AST VII treatment could be related to the further activation of T cells. Difference in the structure alter the activity to stimulate CD4 or CD8 T cell response. DAC-AST VII was more efficient than DC-AST VII in terms of activating $CD8^+$ T cells ($p < 0.05$). Interestingly, DC-AST VII was able to activate higher levels of CD44 on $CD4^+$ T cell than DAC-AST VII. DAC-AST VII is lipophilic compound because of dodecyl and triterpenoid moieties whereas DC-AST VII hydrophilic compound contains carboxyl groups on the structure. We could conclude that lipophilicity/ hydrophilicity could modify the $CD8^+$ and $CD4^+$ T cell response.

Wan et al., revealed that Astragaloside II (AST II), which is the cycloartane type triterpenoid saponin attached 2'-*O*-acetyl-β-D-xylopyranosyl at C-3 and β-D-glucopyranosyl at C-6 on the aglycone, stimulated Con-A and allo-antigen induced T cell proliferation. Also, AST II upregulated CD25 and CD69 activation markers on anti-CD3 induced $CD4^+$ T cells. T cell activation through AST II is regulated by CD45 phosphatase activity and CD45 mediated dephosphorylation of Lyk tyrosine kinase, which is the major intracellular target of CD45 phosphatase activity.[157] CD44 crosslinking can activate Lyk kinase and increase intracellular calcium levels and further activate protein kinase C, which has a role in T cell proliferation.[158] Lyk kinase may have a function in CD44 mediated regulation of AST VII induced T cell proliferation.

Marciani et al., stated that *Quillaja* saponin stimulated cytotoxic T cell proliferation depends on lipophilic acyl side chain.[159] Dodecylamine conjugated QS-21 compound stated as GPI-0100 induced Th1 and CTL response. GPI-0100's lipophilic character may enable it to open a transient pore for delivery of exogenous proteins to cytosolic compartments and the further enhancement of MHC Class I restricted CTL response.[40] QS-21 derivative 3, prepared by modification of the glucuronic acid carboxyl with ethylamine, demonstrated CTL response as QS-21 in mitomycin-C

treated E.G7-OVA cells. QS-21 derivative 2 and 4, prepared by the modification of glucuronic acid carboxyl with glycine and ethylenediamine, stimulated CTL response but not as high as QS-21. Increase in the lipophilicity of the compound influenced CTL response.[160] Conjugation dodecylamine onto glucuronic acid residue increased the lipophilicity of AST VII and further CTL response was improved as shown in Figure 3.25.

Mice vaccinated with ISCOMATRIX/OVA and the number of $CD4^+$, $CD8^+$ T cell were analyzed by FACS. The $CD4^+$ and $CD8^+$ T cell numbers were equal, but CD69 (activation marker) expression percentage in $CD8^+$ T cells was higher than $CD4^+$ T cells.[161] Matrix C treated DCs were also able to enhance cross-present OVA to OT-I cells and increase the CD44 activation marker compared to control.[143] Didierlaurent et al., reported that OVA/ AS01 formulations were injected to mice and MHC II^{high} DCs substantially induced both $CD4^+$ and $CD8^+$ T cell response in T cell: DC ratio dependent manner but $Ly6C^+$ monocytes (which excludes monocyte derived DCs) did not enhance $CD4^+$ and $CD8^+$ T cell response.[107] M1 and M4 (structure defined in Table 3.1.) primed DCs showed higher stimulatory efficiency in an allo-MLR. Effect of M4 on antigen presentation comes from the maturation of hmDC. Moreover, M1 primed and M4 primed hmDC promoted the differentiation of naive T cells into Th1 and the production of IFN-γ by M4.[129]

Sun et al., determined the influence of adjuvant formulations on the ability of DCs to present antigen to DO11·GFP T hybridoma (DO11·10 T hybridoma that recognizes aa 323–339 of OVA in a co-stimulator independent manner). Preparation of OVA in NISV or PLGA microparticles could induce significant levels of antigen presentation by DCs at concentrations, which no presentation was observed with a soluble antigen. This presentation induced by PLGA or NISV was observed in the absence of DCs activation. In contrast, neither the addition of LPS or preparation of OVA in alum could increase antigen presentation to detectable levels. Encapsulation of OVA into an adjuvant nanocarrier system increased its presentation to T cells. Alum, NISV or PLGA have the capacity to stimulate strong antigen-specific immune responses *in vivo*, and even they failed to activate DC. It revealed that their adjuvant effects do not act via DCs interaction as LPS did *in vivo* and there should be other interactions to induce the immune response.[96]

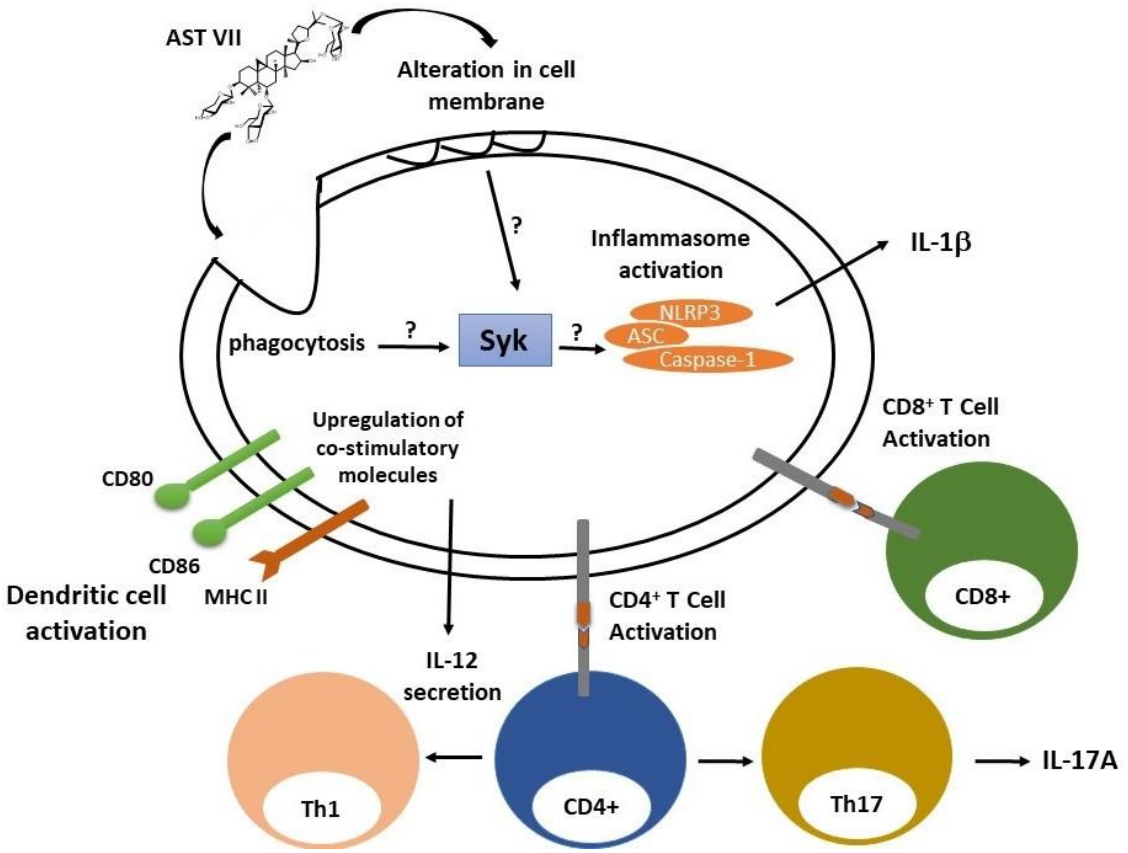


Figure 3.22. Proposed action mechanism of AST VII in dendritic cells (DCs). AST VII could be internalized by phagocytosis or change the dendritic cell membrane structure. Alteration in the membrane could trigger the activation of Syk and further induce inflammasome activation and IL-1β production. Moreover, dendritic cells could be activated by upregulation of MHC II, co-stimulatory molecules (CD86, CD80 etc.) and the production of IL-12. Activated DCs could stimulated $CD4^+$ T cell and $CD8^+$ T cells. AST VII could differentiate $CD4^+$ T cells into Th1 and Th17.

CHAPTER 4

CONCLUSION

Preclinical and clinical studies are in progress to find ideal adjuvant(s)/adjuvant formulations that are able to provide: cellular and humoral immune responses, higher stability, lower side effect, higher bioavailability, easier manufacturing and low production cost. The induction of the innate immune response plays an important role in the activation of the adaptive immune response following the establishment of the first line of defense against a pathological condition. However, the mechanism of actions in innate activation by saponin based adjuvants are poorly understood.

As stated in our previous *in vitro* and *in vivo* studies[55,56,125], AST VII causes Th1 directed strong antibody and cellular immune responses. However, our group has not focused on overriding reason/s of such action; therefore, in this study, we made a preliminary attempt to explain the immunomodulatory mechanism(s) of AST VII. Accordingly, the effects of AST VII on DCs maturation, activation and T cell presentation were studied, whereas two semi-synthetic analogs were synthesized from AST VII based on the encouraging results of the previous reports[162,163] to potentiate the immunostimulatory activity of saponin adjuvants.

On human WB assay, all saponins remarkably stimulated IL-1β response, but Th1 cytokines (IL-2 and IFN-γ) were unexpectedly suppressed contradicting with our previous results.[55,56,125] In BMDCs and BMDMs, co-stimulation of AST VII with LPS produced higher IL-1β titers than the analogs, *viz.* DAC-AST VII and DC-AST VII. DAC-AST VII was the most effective test compound for the production of IL-1β in hWB; however, its activity was not as high as AST VII in BMDCs and BMDMs. It must be emphasized that activities of AST VII and its semi-synthetic derivatives vary on different immune system cells. In addition, AST VII remarkably activated the production of IL-17A compared to DAC-AST VII and DC-AST VII. These results implied specificity of AST VII towards IL-17A production.

As dendritic cell activation is one of the initiation stages of the adjuvants in their immunomodulatory action, it is thought to be a good starting point to study the

mechanism of AST VII. Dendritic cells are professional antigen presenting cells and have crucial role in connecting innate and adaptive immune responses. Dendritic cells can be activated through PAMP dependent (TLR agonist etc.) or PAMP independent manner in response to self molecules, alteration in the microenvironment. NLRs can be also detected by pathogen associated molecular patterns and endogenous danger signals.[89] NLRs detect the PAMPs in the cytosol as well as activated through loss of the cell membrane integrity, insertion of pores and ion channels.[164] To evaluate maturation and activation status of dendritic cells following adjuvants treatments, MHC-II, CD86, CD80 marker expressions and secretion of IL-12 were investigated in BMDCs. AST VII alone did not induce the activation of dendritic cells in terms of expression of co-stimulatory molecules and secretion of IL-12 *in vitro*. It was also shown that AST VII did not activate TLRs alone. Enhanced IL-1β secretion obtained via AST VII and LPS co-stimulation in BMDCs enabled us to investigate the role of LPS-AST VII combination in the maturation and activation of dendritic cells. In the presence of LPS, AST VII was capable of upregulating MHC II and co-stimulatory markers CD86/CD80 and inducing IL-12. Therefore, AST VII is not effective by itself to elicit immunostimulatory activity *in vitro* and requires a co-stimulatory agent such as LPS. The analogs of AST VII (DC-AST VII and DAC-AST VII) increased CD86 and CD80 markers; however, they did not result in MHC II upregulation in dendritic cells. On the basis of remarkable IL-1β secretion, and up-regulation of MHC-II and co-stimulatory molecules following co-treatment with LPS, a synergistic effect of AST VII on TLR/NLR might be the reason in augmentation of the innate immune response. AST VII may have a role in induction of NLRs through the interaction with the cell membrane. AST VII as a triterpenoid saponin, can rearrange the cell membrane organization by intercalating its aglycone backbone in the phospholipid bilayer and extending sugar moieties to the external surface of the membrane. This alteration in the cell membrane can be sensed by dendritic cell as a pathological condition and transduce the signal for its maturation and activation. Moreover, such an effect can be activated Syk kinases to assemble inflammasome and further secretion of IL-1β. To prove this hypothesis, interaction of AST VII with the cell membrane, relevant NLRs and inflammasome activation should be investigated.

Another hypothesis for LPS and AST VII induced IL-1β secretion might be the upregulation of TLR4 on the surface of dendritic cells. As AST IV reduces the IL-1β in

LPS induced epithelial cells through diminishing mRNA expression of TLR4/NF-κB[134], the extra sugar on the AST VII (25-*O*-glucopyranosyl) could result in activation of TLR4 mRNA expression. Thus, the effects of AST VII on the gene transcription and translation of TLR4 and relevant transcriptional factors should be investigated.

After activation, dendritic cells initiate a process for the production of chemokines and cytokines to polarize and activate the T cells subsequently increasing their homing to lymph nodes.[165] To reveal the effects of AST VII and its analogs on T cell presentation, B3Z assay and MLR were performed. In B3Z assay, there was no difference neither in the presence of LPS nor in the absence of LPS in regard to the presentation of OVA following treatment of AST VII and its analogs. Activation of T cells are evaluated by the expression marker CD44. Its upregulation is one of the reasons for TCR engagement and leads to the immune cell migration to the inflammation site. Cross-linking of CD44 with cytoskeleton initiates a process for T cell activation and recruits Src family kinases to transduce the signal. Except being activation and memory markers in T cells, CD44 enhances dendritic cell-T cell tight junction to maintain sustained interaction with TCR and its ligand.[166]

CD44 was upregulated in both $CD4^+$ and $CD8^+$ T cells following treatments of the test compounds in MLR. DAC-AST VII, lipophilic analog of AST VII, was better in the induction of $CD8^+$ T cell response while DC-AST VII (more hydrophilic form of AST VII) was potent in $CD4^+$ T cell response. The lipophilicity of the compound could increase translocation of the antigen to cytosol, leading to more efficient antigen presentation on the MHC Class I molecules to $CD8^+$ T cells, which in turn results in TCR:MHC I engagement and upregulation of CD44.

It is thought that enhanced CD44 expression by AST VII and its analogs imply close contact of DC and T cells in the immunological synapse inducing further T cell activation and polarization via secretion of the cytokines. As CD44 protein expression due to transcriptional activation can be regulated via exposure of cells to pro-inflammatory cytokines such as IL-1β,[167] aforementioned IL-1β secretion from dendritic cells might be the overriding basis for the upregulation of CD44 on T cells. Therefore, further studies are warranted to show the effects of IL-1β on CD44 gene regulation by analyzing its mRNA expression.

Moreover, induction of CD44 on $CD8^+$ T cells following treatment of AST VII and its analogs is crucial because its accumulation enhances the activation of cytotoxic

T cells and subsequently granule exocytosis and cell killing, the main actions of vaccines designed for intracellular pathogens and cancer.[168]

$CD4^+$ T cells have a role in activation of $CD8^+$ T cells and help B cells for producing antibody. Induction of both $CD4^+$ and $CD8^+$ T cell responses initiate a process including the production and secretion of cytokines, chemokines to activate and traffic to immune cells at the infection site and clearance of the pathogen. As weak immunogens, the subunit/recombinant antigens require adjuvants to promote humoral and cellular immunity. However, a few adjuvants have capacity to induce effective cellular immune response. Therefore, the activation data obtained for AST VII and its analog treatments on the $CD4^+$/$CD8^+$ T cells demonstrates the potential of these compounds to be utilized in the vaccines of malaria, HIV and cancer, which require strong cellular immune response.

As discussed above, AST VII and its analogs are potent adjuvant candidates and can be used to induce cellular immunity due to enhanced dendritic cell and T cell activations. Further studies are certainly necessary to shed light into structure-activity relationships, particularly to evaluate the effects of different substituents on the sugar moieties, as well as finding the targets of these compounds in specific gene(s)/transcriptional factor(s)/pathway(s) levels.

Consequently, there is a huge need for adjuvants to potentiate immune responses. Adjuvants for a vaccine are selected in terms of nature of the antigen and its required immune response type. Therefore, understanding the mechanism of action of AST VII is very important to decide its possible use in vaccine formulations that meet the requirements of the antigen to produce an effective and protective immune response against target diseases. This study is a first step to serve the purpose, and subsequent studies towards rational design of AST VII based adjuvant analogs/vaccine formulations to develop prophylactic and therapeutic vaccines are in progress.

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