Characterization of Innate Immune Response to *Nicotiana benthamiana*-derived Influenza H5 Virus-Like Particles

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Abstract

Current influenza vaccine manufacturing processes using chicken-embryonated egg technology is a time-consuming and laborious process, and is currently the major drawback in counteracting pandemic influenza strain. One solution to that problem is the use of plants to generate vaccine antigen. Virus-like particles (VLP), produced from the tobacco plant *Nicotiana benthamiana*, represent a cost-effective, alternative platform for influenza vaccine production.

Previous studies have shown that the immunization with VLP expressing the hemagglutinin (HA) protein from influenza virus H5N1 (H5-VLP) produced in *N. benthamiana*induce protective immunity against challenge of cross-clade virus in mice and ferrets. In this study, we used human peripheral blood mononuclear cells (PBMC) to characterize the innate immune response to plant-derived influenza H5-VLP *ex vivo*. We successfully demonstrate the mitogenic property of H5-VLP on PBMC *ex vivo.* Furthermore, we detect up-regulation of activation marker in B cells and NK cells, and some T cells. Cytokine profile of the supernatant from VLP-stimulated sample suggests that inflammatory response dominates the innate immunity within first 48 hours and is produced by CD14+ monocytes. Our study demonstrates that tobacco plant-derived influenza VLP are capable of generating innate immune responses in naïve human PBMC, helping us to better understand the immunostimulatory nature of this potential vaccine candidate.

Résumé

A l'heure actuelle, la plupart des vaccins contre les infections par le virus influenza sont produits à partir d'œufs de poule fécondés. Ce procédé long et fastidieux constitue l'un des principaux obstacles à la production rapide d'un vaccin lors d'une pandémie. Une solution à ce problème consiste en l'utilisation de plantes afin de générer les antigènes nécessaires à l'élaboration du vaccin. Les pseudovirus ou *Virus-like particles* (VLP) produites à partir de la plante de tabac *Nicotiana benthamiana* représentent une alternative moins couteuse et plus rapide pour la production de vaccins antigrippaux.

Des études préalables ont démontré qu'une immunisation avec les VLP exprimant l'hémagglutinine (HA) du virus influenza H5N1 (H5-VLP) induisaient une immunité protective lors d'une infection par ce virus chez la souris et le furet. Dans notre étude, nous avons utilisé les cellules mononuclées du sang périphérique humain (PBMC) afin de préciser la réponse immunitaire innée suite à l'exposition *ex vivo* aux H5-VLP produites dans *N. benthamiana*. Nous avons démontré les propriétés mitogéniques des H5-VLP sur les PBMC ainsi qu'une activation des lymphocytes B, des cellules NK et de certaines sous populations de lymphocytes T. L'analyse des cytokines sécrétées dans le surnageant des PBMC exposés *ex vivo* aux VLP suggère qu'une réponse pro-inflammatoire prédomine 48h après exposition et semble résulter essentiellement d'une activation des monocytes CD14+. Notre étude démontre que les VLP produites à partir de la plante de tabac génèrent une réponse immunitaire innée dans les PBMC provenant

de patients naïfs, nous permettant ainsi de mieux comprendre les propriétés immunostimulantes de ce nouveau type de vaccin.

List of Abbreviation

aM¦	Alveolar macrophage
APC	Antigen presenting cell
BCR	B cell receptor
CARD	Caspase-recruitment domain
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor 2
CD	Cluster of differentiation
DAMP	Danger associated molecular pattern
DC	Dendritic cell
HA	Hemagglutinin
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
IRF	Interferon regulator factor
IRS-1	Insulin receptor substrate 1
ISCOM	Immunostimulating complex
KIR	Killer-cell immunoglobulin-like receptor
LGP-2	Laboratory of genetic and physiology 2
M1	Matrix 1
M2	Matrix 2
MAP	Mitogen-activated protein
MCP-1	Monocyte chemotactic protein 1
MDA5	Melanoma differentiation-associated gene 5
MHC	Major histocompatibility
MyD88	Myeloid differentiation primary response gene (88)
NA	Neuraminidase
NCR	Natural cytotoxicity receptor
NEP	Nuclear export protein
NFκB	Nuclear factor κB
NK	Natural killer cell

NKT	Natural killer T cell
NLR	Nod-like receptor
NLRP3	NOD-like receptor pyrin domain-containing 3
NP	Nucleoprotein
NSP1	Non-structural protein 1
OSA	Oligoadenylate synthetase
PA	Polymerase acidic Protein
PAMP	Pathogen-associated molecular pattern
PB1	Polymerase basic protein 1
PB1-F2	Polymerase basic protein 1-F2
PB2	Polymerase basic protein 2
pDC	Plasmacytoid dendritic cell
PKR	Protein kinase R
PRR	Pattern-recognition receptor
RD	Repressor domain
RdRp	RNA-dependent RNA polymerase
RIG-I	Retinoid acid-inducible gene I
RLR	RIG-like receptor
RNA	Ribonucleic acid
RNaseL	Ribonuclease L
RNP	Ribonucleoprotein
TGF-²	Transforming growth factor ²
TIRAP	TIR domain-containing adapter protein
TIV	Trivalent vaccine
TLR	Toll-like receptor
TNF	Tumor-necrosis factor
TRAM	Translocation chain-associating membrane protein
TRIF	TIR domain-containing adapter-inducing interferon-β protein
VCAM	Vascular cell adhesion molecule
VLP	Virus-like particle

CHPATER 1: LITERATURE REVIEW

1.1. BIOLOGY OF INFLUENZA VIRUS

1.1.1. History of Influenza Virus

Influenza is an old disease, possibly as old as human evolution. The symptoms associated with influenza infection, including cough, sore throat, and 'peripneumonia', were first documented in Hippocrates' "Epidemics" (vol. VI) the "Cough of Perinthus". However, the term "Influenza" was only introduced in the mid-18th century by Italians, who believed the disease could be attributed to astrological "influences".

Human influenza virus was first isolated in 1933 by Wilson Smith, Christopher Andrewes, and Patrick Laidlaw at the National Institute for Medical Research in London, England. This first clinical isolate was used to infect ferrets, which developed influenza-like symptoms. Furthermore, these investigators showed that the virus could be transmitted back to humans from infected animals. This landmark study not only established that influenza virus was the etiologic agent of clinical influenza, but also that it was capable of transmission between animals and humans [1].

1.1.2. Influenza Viruses in Ecosystem

The role of wild animals is considered extremely important to the biology and epidemiology of influenza. In fact, it is generally accepted that wild birds, especially from the Orders Anseriformes and Charadriiformes (aquatic avian species such as ducks and geese), are the natural reservoirs for influenza viruses

[2,3]. This observation has several implications: a) since virus is excreted in the feces of aquatic birds, water becomes an important medium for transmission; b) animals in contact with contaminated water are likely to be exposed to and co-infected with multiple strains of influenza viruses, thus providing opportunities for viral genome re-assortment (discussed below); c) the long migratory routes and distinctive migratory patterns of these birds are likely to be major factors in the global movement of these viruses. Influenza viruses also infect a number of other avian (eg: chickens, turkeys) and mammalian (eg: pigs, cats) species that live in close proximity to humans. For these reasons, as well as the intrinsic genetic instability of this family of viruses, it is extremely difficult to predict which strain(s) of influenza virus will emerge to cause the next seasonal outbreak or the next pandemic and to formulate effective control strategies. Although a better understanding of influenza virus ecology may provide some insight into viral evolution [4], it is unlikely that an adequate 'primary control strategy' can ever be devised for these viruses.

1.1.3. Viral Genome, Proteins, and Structure

There are three types of influenza viruses circulating in nature: influenza A, B, and C. Influenza A and B viruses share genomic and structure similarities, while influenza C virus has a smaller genomes and encodes less proteins [5]. Nonetheless, all three influenza viruses are capable of infecting human and cause disease. Due to the relevance with our work, the description of the viral structure will be based on influenza A virus.

The genome of influenza viruses contains eight segmented, negative-sense RNA molecules. These RNA encode 11 viral proteins: hemagglutinin (HA), neuraminidase (NA), matrix 1 and 2 (M1 and M2), nucleoprotein (NP), non-structural protein 1 (NSP1), nuclear export protein (NEP), polymerase acidic protein (PA), polymerase basic protein 1 and 2 (PB1 and PB2), and polymerase basic protein 1-F2 (PB1-F2).

Influenza virions bud from the host plasma membrane and take a spherical shape. The viral envelope is derived from the host cell's plasma membrane and contains HA, NA, and M2. Sitting beneath the viral envelope is a matrix of M1 protein that holds the viral ribonucleoproteins (vRNPs) in place. vRNPs at the core of the virus consist of viral RNA and the proteins NP, NEP as well as the viral RNA polymerase complex (PB1, PB2 and PA) (**Figure 1**).

Both HA and NA are used in subtyping of influenza viruses. At present, there are 17 types of HA and 10 types of NA identified [6]. The combination of HA/NA determines the subtype of the virus, making a total of at least 170 possible influenza subtypes circulating in nature. Within each of these strain designations (eg: H1N1), there can also be large numbers of more-or-less distinct sub-types (termed clades and sub-clades) based upon the accumulation of mutations in the HA and NA genes as well as other viral proteins. Currently, only influenza subtypes H1N1 and H3N2 are actively circulating among the human population [6]. This situation is virtually certain to change; however, this only occurs with the periodic introduction of HA or NA genes from non-human

influenza strains (i.e. avian viruses) through the infection of intermediate domestic animal hosts (e.g. chickens, pigs; discussed below).

1.1.4. Influenza Virus Life Cycle

The first step of viral replication cycle is entry into the host cell. Receptor-mediated entry of influenza is wholly dependent on the HA protein. Homotrimeric HA forms as a complex on the viral surface, consisting of three HA0 monomers. HA0 has two functionally distinct subunits: HA1, which contains the receptor binding domain, and HA2, which has the fusion peptide. Binding of the HA1 subunit to the host neuraminic acid (or sialic acid) facilitates viral entry through receptor-mediated endocytosis [7]. Alternatively, influenza virus can enter host cells by macropinocytosis [8] (**Figure 2A**).

The acidic environment of the endosome plays a key role in releasing the viral genome into the host cell. At low pH, HA0 undergoes conformational change, exposing the HA2 fusion peptide while maintaining HA1 binding [9]. HA2 inserts itself into the endosomal membrane so that the viral envelope and the inner leaflet of the endosomal membrane can be brought into proximity and subsequently fuse, exposing the interior of the virion to the host's cytoplasm.

In addition to inducing HA0 conformational change, the acidic environment of the endosome also has an effect on the M2 protein. M2 is a proton-selective ion channel protein that allows influx of protons from the endosome into the virus particle [10,11]. This acidification of the virion disrupts the interaction between M1 and vRNPs, resulting in expulsion of the vRNP into

host cell's cytoplasm (**Figure 2B**). The vRNP contains a nuclear localization signal that mediates its transport into the nucleus using cellular machinery [12].

The viral genome is composed of eight negative-sense RNAs; thus, the native RNAs must be converted into positive-sense RNAs to serve as templates for the purposes of translation and replication. Transcription of the viral genome requires a primer for initiation. All viral mRNAs (vmRNA) are 5'-capped and 3'-polyadenylated in a manner that is host machinery-independent. First, the 5' cap is hijacked from cellular mRNA by an endonuclease activity of PB1. PB1 cleaves 10 to 15 nucleotides 3' of the cap structure and uses it as a primer for vmRNA synthesis (cap snatching). Next, the viral RNA-dependent RNA polymerase complex (vRdRp; consists of PB1, PB2, and PA) transcribes, elongates and synthesizes the poly-(A) tail. Elongation of the vmRNA is halted when the vRdRp complex encounters a stretch of 5 to 7 U residues (signal for polyadenylation). The vRdRp complex moves back and forth to transcribe this stretch of U residues repeatedly, leading to the formation of a poly-(A) tail. The resulting vmRNA is transported to cytoplasm for translation (**Figure 2C**).

Unlike the generation of vmRNA, replication of the viral genome is primer-independent and only requires the vRdRp complex. Once the full-length complementary RNA (cRNA) is produced, it serves as a template for progeny viral RNA (**Figure 2D**). Currently the "switch" signal between cRNA and vmRNA production is not well understood, but it has been proposed that the accumulation of soluble NP and vRNP plays a role in the process [13,14].

The apical side of polarized respiratory epithelial cells marks the location of viral assembly and budding [15]. Viral surface proteins, HA, NA, and M2 are transported from the endoplasmic reticulum to the plasma membrane through the Golgi apparatus. In contrast, migration of vRNPs is less well understood, although it has been suggested that M1 exploits the cytoskeletal network to guide vRNPs to the assembly site [16]. Furthermore, M1 is required for the final closing and formation of spherical viral particles [17]. The neuraminidase (NA) activity of the newly assembled virion cleaves off any linkages between HA and host membrane sialic acid residues after budding, thus releasing the viral particle to begin a new cycle of infection and replication (**Figure 2E**).

1.1.5. Epidemiology, Transmission and Pathogenesis

Seasonal influenza is estimated to infect roughly 5 to 20% of the human population each year [18]. In temperate regions, these infections mostly occur in autumn and winter. The virus can infect people of all ages, although the prevalence of symptomatic disease is greatest in the young and the elderly. Severe illness and death are usually seen in people over the age above 65 or with serious co-morbidities. Children generally have the highest rates of infection and shed the most viruses [19].

Repeated influenza infections in the same individual over time can be largely attributed to two processes in the virus known as antigenic drift and antigenic shift. So-called drift variant of viruses are usually the cause of seasonal influenza and arise due to the lack of proof-reading activity of the vRdRp. The

low-fidelity of vRdRp complex leads to spontaneous mutations in the genetic sequences of viral surface proteins (mainly HA and NA) at an estimated rate of $1x10^{-3}$ to $8x10^{-3}$ mutations per year [20]. While pre-existing humoral and cellular immunity can often protect the host from re-infection with the same virus, drift variants that are no longer well recognized by the immune system can sometimes escape neutralization. These drifted viruses are thus selected and can then spread in the human population.

Occasionally, when two or more strains of influenza viruses co-infect the same host cell, the viruses can undergo genetic re-assortment and produce progeny that contain gene segments originating from both parental strains. Such genetic re-assortment is known as antigenic shift, and is often the cause of the introduction of new viral strains from animals into the human population [21]. Domestic animals such as pigs are an excellent example of “mixing vessels” in which antigenic shift can occur. Pig lung epithelial cells express both ±-(2,6) linked sialic acid moieties (the receptor for human influenza strains) and ±-(2,3) linked sialic acid moieties (the receptor for avian influenza strains). Having both receptors dramatically increases the risk of simultaneous infection by both human and avian strains in a single cell with a resultant increase in the chance of re-assortment [22]. Because of the lack of collective immune memory, a newly emerged influenza virus that has undergone an important antigenic shift has the potential to cause a serious epidemic or, on a larger scale, a pandemic.

There are several ways that influenza viruses can be transmitted: by exposure to aerosolized droplets from infected patients; by contact with

contaminated objects (fomites); or by direct contact with contaminated surface of individual (eg: handshake) [23]. In humans, influenza infection causes a broad spectrum of manifestations, from asymptomatic infection to serious illness with a wide range of complications. During the acute infection, patients may experience cough, myalgia, sore throat, malaise, and other non-specific symptoms, which typically persist for 7 to 10 days. The most common complications of influenza infection include primary viral pneumonia and secondary bacterial pneumonias [24]. Recently, a rare but severe complication characterized by excessive immune response termed 'cytokine storm' has been recognized in healthy individuals who deteriorate rapidly after infection [25]. Neuromuscular and cardiac complications of influenza can occur but are very rare. In the most severe cases, influenza infection can lead to death.

Influenza viruses establish the primary site of infection in the epithelial cells of the upper and lower respiratory tracts. Although influenza RNA has been detected in the blood in severe disease [26], viremia is not thought to be a common occurrence. The peak viral load in the lungs is thought to occur on day 2-3 after inoculation and decreases steadily thereafter, correlating well with the typical evolution of clinical symptoms [27]. Destruction of ciliated columnar epithelial cells is common and can be caused by local mucosal inflammation [28] as well as the direct cytopathic effects of the virus [29]. Complete viral clearance usually occurs by two weeks post-infection, but full tissue repair and resolution of influenza-associated lung pathology may take up to 1 month.

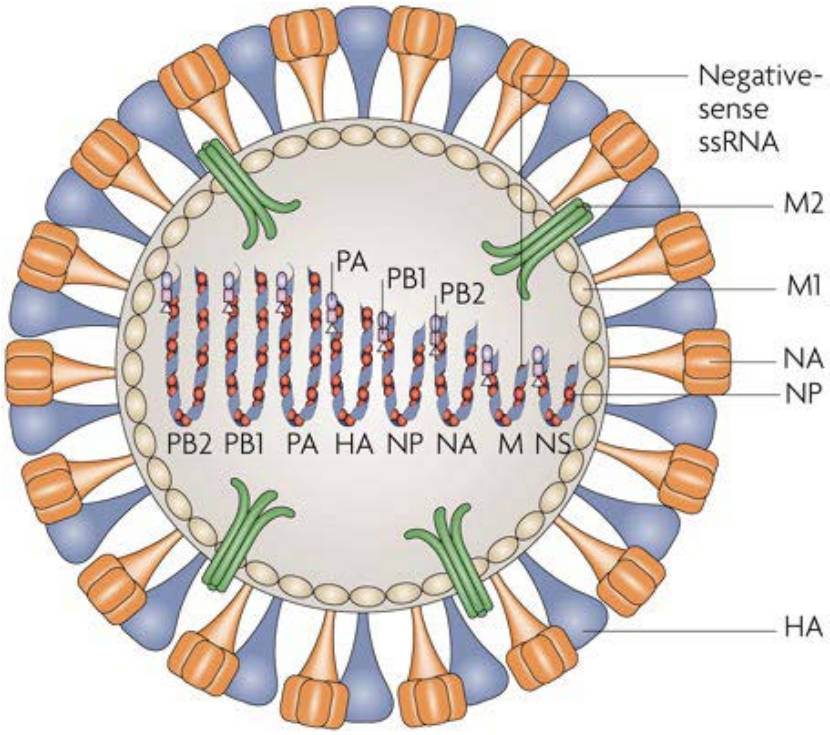

Figure 1: Influenza Virus Structure and Genome Arrangement. (Adapted from [30]). The structure of a prototypic influenza virus can be divided into two parts: the envelope and the capsid. The viral envelope consists of a lipid bilayer derived from host cell plasma membrane and has the viral HA, NA and M2 proteins embedded in it. These surface proteins play a crucial role in both viral entry into and budding from the host. Immediately beneath the viral envelope is the M1 protein, which form the outer capsid of the virus. Within the capsid are NS1 (regulating transcription and replication) and NEP (transporting viral genome) proteins, as well as the 8 segmented viral RNAs that are tightly associated viral proteins PB1, PB2 and PA. PB1, PB2 and PA are responsible for transcription and translation of the viral genome.

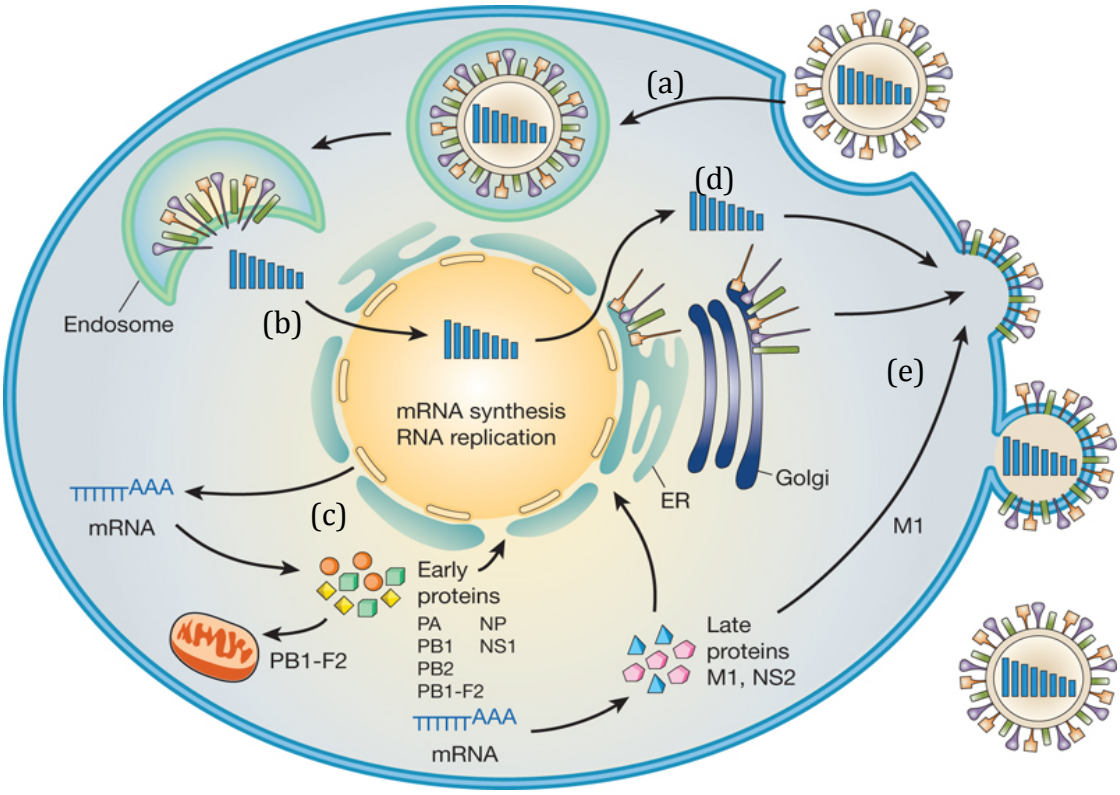

Figure 2: Influenza Virus Life Cycle (Adapted from [31]). (a) Viral HA proteins bind to host surface sialic acid residues to facilitate viral entry through endocytosis. (b) Acidification of the endosome leads to transfer of the viral genome into the cytosol, which is then transported into the nucleus. (c) Viral RNAs are transcribed into vmRNA in the nucleus followed by transport into the cytosol for viral protein synthesis. (d) Full length (genomic) viral RNAs are also replicated in the nucleus and transported to the cytosol for assembly. (e) Viral proteins and RNAs are assembled on the apical aspect of polarized host respiratory epithelial cells. Viral NA on the newly-formed virions cleaves the linkage between HA and sialic acid to release the virions to the external environment.

1.2. HOST INNATE IMMUNE RESPONSE TO INFLUENZA VIRUS

Innate immunity is an early defense mechanism against invasion of microorganisms, including influenza virus. One powerful innate defense against all respiratory viruses involves the secretion of mucus by epithelial cells in the respiratory tract and the concerted action of cilia to expel unwanted materials and visitors – a physical barrier. Another layer of defense occurs at the level of the respiratory epithelial cells as well as lung-resident immune cells (eg: alveolar macrophages, dendritic cells). Disease can occur when viruses evade or overwhelm the innate response to establish local infection or by an overactive or misdirected innate responses.

1.2.1. Pattern Recognition Receptors

Recognition of pathogens in the early stage of infection is critical in initiating the host response against infection. This process relies on the detection of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) [32]. Signal transduction through PAMP-activated PRRs leads to activation of innate immune responses. At the current time, several different 'families' of PRR have been described.

1.2.1.1. Toll-Like Receptors

Toll-like receptors (TLRs) are transmembrane proteins consisting of an "internal" signal transducing domain, a transmembrane domain, and an "external" domain responsible for PAMP recognition [33]. Currently there are 10 TLRs

identified in humans, which can be classified into two groups depending on their location of expression. TLR1, TLR2, TLR4, TLR5, and TLR6 are cell membrane-bound TLRs that recognize microbial surface components. In contrast, TLR3, TLR7, TLR8, and TLR9 reside in various intracellular compartments and recognize foreign nucleic acids [32,34]

Currently there is no evidence of innate immune activation through recognition of influenza viruses by surface TLRs; however, activation of intracellular TLRs (TLR3 and TLR7) in response to influenza virus infection is well documented [35,36].

TLR3 recognizes double-stranded RNA (dsRNA) of different sources such as poly-(I:C), dsRNA virus (reovirus), and ssRNA viruses during genomic replication [37]. In contrast, TLR7 can be activated by single-stranded RNA that is present throughout the virus life cycle [36]. Signaling through TLRs recruits adaptor proteins, MyD88, TRAM, TRIF, and TIRAP. Two major TLR signaling pathways have been characterized to date: MyD88-dependent and TRIF-dependent [33]. MyD88-dependent signaling is observed in downstream of all TLRs except TLR3; its engagement activates transcription factors, NFº B and AP-1, which lead to the expression of inflammatory cytokines including IL-1, IL-6 and TNF-± [38]. The TRIF-dependent pathway is activated through TLR3 and TLR4 signaling, which leads to expression of the powerful transcriptional factor IRF-3, and subsequent expression of IFN-² [39] (**Figure 3**).

1.2.1.2. NOD-Like Receptors

NLRs are intracellular PRR that contain C-terminus leucine-rich repeats that facilitate antigen recognition, an N-terminus PYRIN domain responsible for interacting with downstream signaling molecule, and a central nucleotide-oligomerizing domain. There are currently more than 20 NLRs under investigation, all of which respond to PAMP and danger-associated molecular pattern (DAMP) stimulation by increasing the expression of inflammatory cytokines through MAP kinase and NFº B signaling [40,41].

The mechanism of NLR activation in response to influenza virus infection is not well understood. One report suggest that NLRP3 inflammasome can recognize viral RNA leading to the production of functional IL-1² [42] another study suggests that NLRP3 inflammasome activation is the result of disturbance in the intracellular ionic concentration caused by the presence of influenza M2 ion channels [43]. These findings imply that there may be multiple mechanisms by which NLR contribute to the rapid recognition of influenza viruses.

1.2.1.3. RIG-I-Like Receptors

RIG-I-like receptors (RLR) are a family of cytosolic PRR that consist of at least three members: retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and LGP-2. These RLRs have a DExD/H helicase domain that functioned as RNA sensor, and a repressor domain (RD) at their C-terminus that inhibits downstream signaling. However, only RIG-I and MDA5 contain caspase-recruitment domain (CARD) at their N-terminus that

is responsible for signaling through IRS-1 adaptor protein and the production of pro-inflammatory cytokine and type-I interferon expression [44]. Because of the absence of CARD domain in LGP-2, LGP-2 was suggested to negatively regulate RNA virus-induced response by binding to RD domain of other RLR members [45].

RIG-I and MDA5 share structure similarity and both recognize dsRNA. However, previous studies have shown preferential activation of these cytosolic sensors by different RNA viruses. For example, RIG-I is known to recognize members of the family *Paramyxoviridae*, Japanese encephalitis virus, and influenza virus [46], while MDA5 is essential for the recognition of members of *Picornaviridae* such as encephalomyocarditis virus, and theilovirus [46,47] (**Figure 3**). The helicase domain of RIG-I has been shown to recognize 5' triphosphate of RNA [48], while there is currently no evidence showing similar observation in MDA5. Furthermore, Kato et al illustrates that differential activation of RIG-I and MDA5 by dsRNA may be length-dependent [49]. Despite these differences, RIG-I and MDA5 nonetheless provide important defense mechanism during RNA viral infections.

1.2.2. Cytokines

One of the major results of signaling via PRRs is the orchestrated production of a well-defined set of cytokines that act as both local and distant effectors. Cytokines are signaling molecules secreted by various cells in response to an activating stimulus; they are involved in all stages of the host response to

infection. Cytokines can be roughly grouped based on their biological activities. For instance, some cytokines such as IL-1² and TNF-± tend to promote inflammation, hence termed “pro-inflammatory cytokines”; others like IL-4 and IL-10 tend to suppress inflammatory responses and are called “anti-inflammatory cytokines”. When influenza virus activates innate immune defenses by stimulating PRRs such as TLRs and RIG-I, this leads to the secretion of both type-I interferon and pro-inflammatory cytokines that participate in the immediate antiviral response.

1.2.2.1. Type-I Interferon and Antiviral Response

Type-I interferons are transcriptionally activated upon viral infection; they initiate potent innate defense mechanisms against viral infection. Although several type-I interferons have been identified, only the IFN-± and IFN-² exhibit antiviral properties [50,51]. These anti-viral type-I IFNs regulate the expression of IFN-stimulated genes that specifically target viral replication pathways. For example, IFN-±/² signaling leads to increasing expression of protein kinase R (PKR), which is responsible for inhibiting cellular translation machinery, thus halting viral protein synthesis [52]. IFN-±/² signaling also up-regulates expression of 2’-5’ oligoadenylate synthetase (OAS). The oligonucleotide generated by OAS (2’-5’A) directly activates RNaseL, which can degrade both viral and cellular RNA [53,54]. Most importantly, IFN-±/² strongly promote functional maturation of antigen presenting cells through up-regulation of MHC-I and II, as well as co-

stimulatory molecules CD80 and CD86, which enhance the communication between innate and adaptive immunity [55,56].

1.2.2.2. Pro-Inflammatory Cytokines

Pro-inflammatory cytokines are typically produced at the early stages of response to a microbial challenge and contribute to both innate and adaptive immunity. The "classical" pro-inflammatory cytokines, IL-1±/² and TNF-±, have a wide range of functions: they up-regulate adhesion molecules such a VCAM, ICAM-1 and E-selectin on vascular endothelial cells [57,58,59]; they stimulate the production of chemokines such as IL-8 and MCP-1 by endothelial cells [60,61,62]; they can also induce the expression of phospholipase A2, cyclooxygenase-2 and inducible NO synthase, which produce prostaglandins and nitric oxide that cause vasodilation and increase vascular permeability [63,64,65]. On the other hand, the production of pro-inflammatory cytokines can markedly worsen the pathology associated with viral infection. For example, when some young and healthy patients are infected with highly pathogenic influenza virus – cytokine-induced influx of circulating leukocytes can exacerbate local inflammation, which leads to pathology: so-called "cytokine storm" [25,66,67,68].

1.2.3. Cellular Response In Influenza Virus Infection

Influenza viruses enter the host through the respiratory tract and establish primary infection in respiratory epithelial cells [69,70]. Many cells participate in

the initial response to influenza virus invasion. Local antigen presenting cells (APC), such as macrophages and dendritic cells (DC), are powerfully activated by the virus through recognition of PAMPs (eg: viral RNAs) and DAMPs (eg: heat-shock proteins, galectins [71]; Natural Killer (NK) and NKT cells are responsible for early control of virus infection through cytokine secretion and direct cytolysis. APCs serve two major purposes in this setting: 1) the capture, processing, and presentation of antigen in a recognizable form to T cells; and 2) to provide the signals necessary for the maturation of the adaptive response (eg: cytokines and chemokines).

1.2.3.1 Role of Alveolar Macrophages

In contrast to the 'classical' phagocytic function of macrophages, resting alveolar macrophages (aM¦) are considered to be primarily regulatory in nature, with low production of inflammatory cytokines, low levels of phagocytic activity [72], and in the right conditions, active production of IL-10, TGF-², nitric oxide and prostaglandins to suppress T cell activation [73]. During influenza infection however, aM¦ become active phagocytes and are able to produce high level of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-± [74], which promote infiltration of circulating monocytes to the site of infection through up-regulation of the chemokine receptor, CCR2 [75]. These infiltrating monocytes differentiate into either DCs or mature macrophages that function as APCs and/or sources of even more pro-inflammatory cytokines [76]. Although aM¦ activation can ultimately lead to lung pathology in the context of influenza infection, animals

deficient in aM¦ are unable to mount an adequate virus-specific CD8 T cell response and have increased mortality [77,78]. It has also been proposed that aM¦ limit virus spread by phagocytosing apoptotic epithelial cells as well as processing viral antigen for presentation to T cells to initiate the adaptive immune response [79,80].

1.2.3.2. Role of Dendritic Cells

Dendritic cells (DCs) play a crucial role in bridging innate and adaptive immunity. They are normally distributed in relatively low numbers throughout the respiratory tract where they provide surveillance for invading pathogens [81]. Several different classes of DCs are found in the influenza-infected lung, including local airway (resident) DCs, infiltrating monocyte-derived DCs (mDC), and plasmacytoid DCs (pDC).

Development of the adaptive antiviral response requires DCs to acquire and present viral antigen to T cells in the context of MHC molecules [82]. DCs acquire influenza viral antigens in two ways: 1) through direct infection by the virus [82,83] and 2) through phagocytosis of viral-induced apoptotic epithelial cells [84,85]. Both routes permit the presentation of viral antigen on MHC-I, even though the mechanisms are different. Virus-infected DCs process *de novo* synthesized viral protein using proteasome machinery, deliver antigen to endoplasmic reticulum where it is loaded onto MHC-I [86]. In contrast, DCs that phagocytose apoptotic cells that would normally present viral antigens on MHC-II

can cross-present antigens on MHC-I when appropriate stimuli are present (ie: cytokines, PAMP-PRR signaling) [87,88].

The migration of antigen-bearing DCs to local draining lymph node (LN) is important for the initiation of adaptive immunity, and failure to migrate results in a significant reduction in virus-specific CD4 and CD8 T cell responses [89]. Migration of DCs to the regional LN depends on expression of the chemokine receptor, CCR7, and its ligands, CCL19 and CCL21 [89,90].

The different subtypes of DC likely play complimentary roles in influenza virus infection. For example, pDC produce large amounts (even more than conventional DCs) of type-I interferon in response to influenza infection [36,91]. However, unlike conventional DCs and monocyte-derived DCs, it appears that pDC do not present viral antigen to T cells [92]. Instead, pDC may predominantly promote antibody production by plasma cells through secretion of type-I interferon and IL-6 [93]. Both pDC and monocyte-derived DCs also contribute to antiviral response through the expression of high levels of IL-12, which promotes the differentiation of naïve T cells into Th1-type cells that are critically important for targeting infected cells [94].

1.2.3.3. B cells

In addition to their role as producers of antibodies, conventional B cells that express the classical B-cell receptor (BCR) are capable of recognizing their cognate antigen. Binding of this antigen can lead to receptor-mediated endocytosis and MHC-II-dependent antigen presentation to T cells. This

phenomenon has been observed in the context of certain influenza HA subtypes [95]. B1 cells are a limited-repertoire subtype of B cells that exhibit innate-like qualities. During acute influenza virus infection, $CD5^+$ B-1a cells can markedly increase production of IgM [96] that is capable of neutralizing the virus. In addition, this IgM can couple with complement to initiate viral lysis [97]. Unlike conventional B cells, B1 cells do not undergo clonal expansion during viral infections including influenza; instead, they are recruited to the site of infection to mediate immediate protection [98].

1.2.3.4. Natural Killer Cells

Natural killer (NK) cells are innate effector cells that defend against both invading pathogens and malignant transformation of host cells. In humans, NK cells have traditionally been identified simply as $CD56^+$, $CD3^-$. However, subsequent studies have subcategorized NK cells based on the level of CD56 expression: $CD56^{bright}$ NK cells which represent a minority of the peripheral NK cell population mainly produce cytokines while $CD56^{dim}$ NK cells are more numerous and function as cytolytic effector cells. The balance between activating and inhibitory signals on host cells constantly regulates NK cell activity. An activating signal can be generated in several ways including the binding of activation receptors (eg: natural cytotoxicity receptors 'NCRs' and Fc receptor CD16) to their ligands, through cytokine signaling by type-I interferons, IL-12 and IL-18, or through loss of an inhibitory signal [99]. NK cells also express

inhibitory receptors (eg: KIRs and NKG2A/CD94 complex) that regulate their activities at resting state [99].

The NK cell-mediated antiviral response is necessary for control of influenza virus, as deficiency in NK cell activity usually results in increased morbidity and mortality shortly after infection [100]. This early protection can be mediated by several mechanisms. For example, the engagement of NKp44/ NKp46 (members of NCR family) and NKG2D to their ligands (influenza HA proteins and inducible stress protein ULBP, respectively) on the infected target cells [101,102] can activate NK cells. Alternately, IFN-± produced by influenza virus-activated DCs enhance the cytotoxic activity of NK cells and infected DCs can promote IFN-Ò production by NK cells through secretion of IL-12 [103].

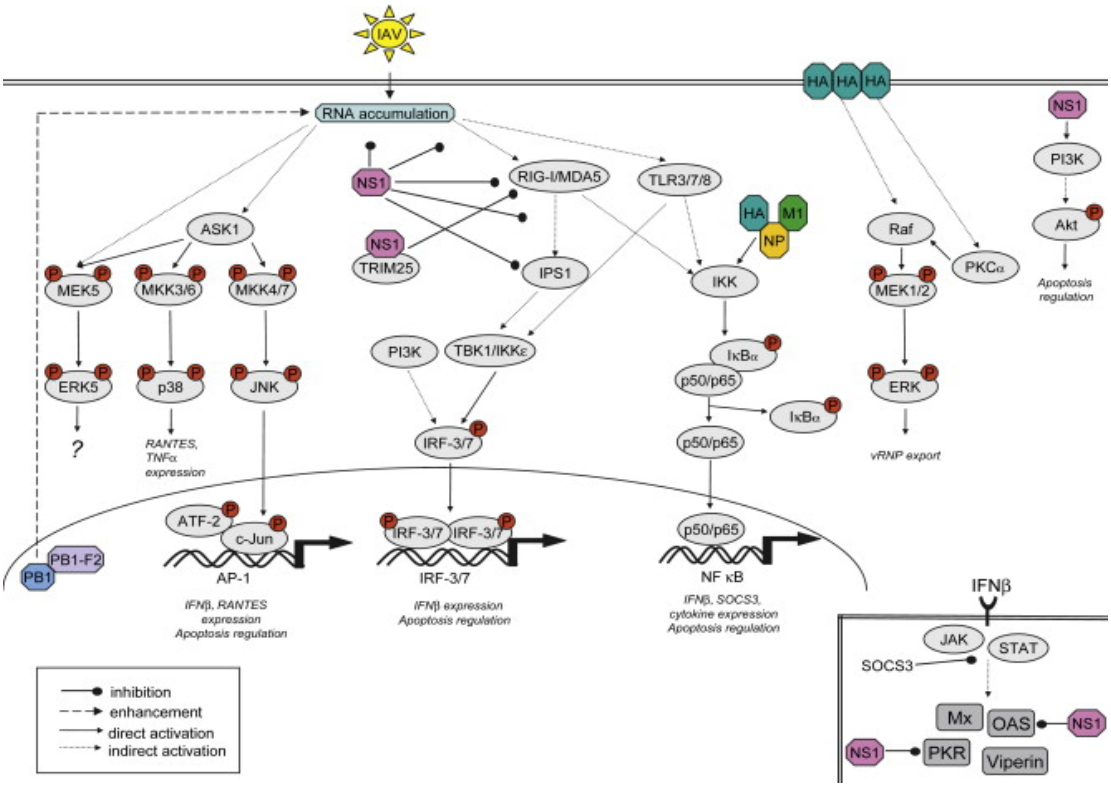

Figure 3.1: Innate Immune Signaling to Influenza Virus (Adapted from [104]). Several intracellular innate sensors are capable of detecting the presence of influenza RNA. These include intracellular TLR3 and TLR7 that recognize influenza virus dsRNA and ssRNA respectively. Signaling through TLR3 and TLR7 triggers an NFº B-dependent inflammatory response and IRF3-dependent anti-viral response. Similarly, RIG-I-like receptors such as RIG-I and MDA5 can be activated by influenza RNA, triggering the production of type-I interferons (IFN-±).

1.3. INFLUENZA VACCINES

The apparently endless re-emergence of influenza virus variants is a serious global health concern. Each year, these viruses not only cause immense economic losses, they also causes significant morbidity and mortality in human populations. One potential solution to this problem is the introduction of influenza vaccines. Ernest W. Goodpasture and colleagues at Vanderbilt University first reported successful growth of influenza virus in embryonated hens' eggs in 1931, which later became the main method of virus production and preparation of vaccine antigens. In the last 80 years, embryonated hens' eggs have been the main source of viral antigen for the production of influenza vaccines. However, this manufacturing platform has raised concerns regarding its response time and surge capacity efficiency in the event of influenza pandemic. In 2009, type-A porcine H1N1 (pH1N1) influenza virus emerged and posed a serious threat due to the absence of cross-protective immunity from the past seasonal influenza vaccines or exposure to circulating wild-type H1N1 strains [105,106]. Indeed, only those born before 1957 had any evidence of cross-reactive immunity to the new pH1N1 strain [21]. It was speculated that both the absence of prior immunity, as well as the delayed production of an appropriate vaccine contributed to the rapid global spread of this virus. Although the 2009-10 pH1N1 pandemic was relatively mild, it moved the issue of rapid and 'scalable' vaccine manufacture far up the list of influenza-related public health priorities [107].

1.3.1. Current Global Influenza Vaccine Manufacturing

Influenza vaccines are still predominantly manufactured using embryonated-egg technology. In short, the Global Influenza Surveillance Network (GISN) tries to predict the three most dangerous strains (an H1N1, an H3N2 and a B strain) for inclusion in the upcoming year's seasonal trivalent vaccine (TIV). The three strains chosen may differ between the northern and southern hemispheric versions of the vaccine. After initial identification, the selected strains must be adapted for optimal growth in eggs. Typically, the targeted viruses are injected into the amniotic cavity of fertilized chicken eggs along with an egg-adapted strain (e.g.: PR8). Because the gene segments of influenza viruses segregate independently, progeny viruses include all possible viable genetic combinations [108]. The most promising candidate viruses are recovered from the amniotic fluid, verified and amplified. In addition, these vaccine strains can be attenuated (genetically-modified for optimal growth at temperatures below 37°C), or inactivated (UV-light, or chemical treated viruses). This whole process typically takes up to 6 months to manufacture sufficient numbers of doses for large populations [109].

1.3.2. Preparation of Influenza Vaccines

There are two main types of egg-based influenza vaccines on the market today: live-attenuated vaccines and inactivated vaccines. Live-attenuated vaccines are based upon genetically modified influenza strains (cold-adapted) that replicate sub-optimally at the human core body temperature [110]. Live-attenuated

vaccines are well tolerated and are usually recommended for subjects between 2 to 49 years of age. The immune responses induced by the attenuated vaccine are similar to those seen following natural infection [111]. It was initially believed that a single dose of the live-attenuated vaccine would induce protective immunity [112,113] but more recent studies suggest that single dosage may only provide sub-optimal protection, particularly in young children [114,115]. In terms of safety, there is a general concern regarding live-attenuated vaccines due to the possibility of genetic reversion or recombination that could allow restoration of virulence, as in the case of polio vaccine, or confer entirely new properties [116,117]. Inactivated vaccines use wild-type viruses (a source of biohazard concern at the site of manufacture) and are usually prepared in a two-step process. The first step is to kill the virus, which is accomplished by chemical treatment such as formaldehyde, or UV light treatment. After inactivation, the virions are treated with detergent such as Triton® X-100 to generate split virions and viral protein subunits (eg: concentrated surface glycoproteins). This process destroys any remaining infectivity and overall structure. Inactivated viruses cannot replicate in the subjects; however, they tend to elicit weaker immune responses and can require an adjuvant to develop protective immunity [118].

1.3.3. Influenza Vaccines and Adjuvants

Adjuvants are compounds or macromolecular complexes that enhance the ability of an antigen to induce an immune response. Adjuvants are often added to vaccines to achieve a desired immune response (i.e. more Th2-mediated antibody

production or more Th1-medidated cellular immunity) and/or to reduce the amount of antigen used per dose [119]. Different adjuvants have different mode of actions but in general can be divided into two classes: immunostimulants and vehicles. The former directly interact with immune system to increase responses to antigens. For example, monophosphoryl lipid A is a derivative of lipopolysaccharide that targets TLR-4 activation [120,121]. The latter promote optimal antigen presentation thereby favoring the development of an appropriate immune response. In some respects, aluminum-based salts (alum) are 'vehicle-type' adjuvants because they can bind to the cell membrane of DCs and enhance antigen presentation to Th2 CD4 T cells that support antibody responses [122]. Although influenza antigens have historically been administered without adjuvants, the use of adjuvants has advanced rapidly in recent years, even more so by the widespread use of adjuvanted pH1N1 vaccines in many parts of the world. Oil-in-water adjuvanted vaccines (eg: the MF59 adjuvanted Novartis TIV) are now widely recommended for elderly subjects [123]. Among many other studies conducted during the pH1N1 pandemic, Ferguson et al. demonstrated that AS03-adjuvanted monovalent A/California/07/09 vaccine (GlaxoSmithKline) induced higher titre antibody responses and greater seroconversion rates than antigen alone [124].

1.3.4. Efficacies of Current Influenza Vaccines

Current influenza vaccines are based on the technology developed in mid 20th century. Although the vaccines are able to elicit anti-influenza immune

response, a recent meta-analysis on the traditional inactivated influenza vaccines, made from embryonated hens' eggs, revealed that only 59% of subjects between ages of 18 and 65 years developed neutralizing antibody response after immunization [125]. The same group also analyzed the efficacy of live attenuated influenza vaccines and found 83% of children between ages of 6 months to 7 years were protective. Since inactivated vaccines are somehow ineffective and there are concerns regarding safety of live attenuated vaccines (despite being effective), new type of vaccines should be developed to overcome these drawbacks.

1.4. VIRUS-LIKE PARTICLES (VLPs)

1.4.1. Introduction to VLPs

In the last decade, the advancements of both DNA recombinant technologies and genetic engineering have led to the development of many candidate subunit vaccines. Subunit vaccines are generated by expressing genetic sequence of the specific component of the pathogen in a heterologous system, such as yeast or mammalian cells. These vaccines are safe, but often suffered from poor immunogenicity due to improper protein folding and/or other modifications (eg: glycosylation). Such subunit vaccines often require higher doses, multiple boosters, and/or co-administration of adjuvants [126,127].

Virus-like particles (VLPs) are a nanoparticulate of sub-unit vaccines that 'look like' the targeted viral pathogen in many respects. VLPs are generally devoid of genetic material, thus making them non-infectious and relatively safe. Unlike other subunit vaccines however, VLPs retain many structural and morphological similarities with their authentic pathogen counterparts. As a result, VLP-based vaccines are often able to elicit immune response that are closer to those observed following natural infections [128].

Several different types of VLPs exist that can be distinguished by how the particles are constructed.

1. Classic VLPs

The classic VLPs (cVLP) contain one or more viral antigens and auto-assemble after expression to form a well defined, stable structures that preserve

the native antigenic conformation of the immunogenic proteins without the incorporation of any viral genetic material [129]. For instance, the major HPV capsid protein, L1, can be expressed using recombinant technology and self-assembles to form HPV-like particles. These particles are highly immunogenic and are approved by Food and Drug Administration as vaccine to prevent HPV infections [130].

2. Virosomes

Virosomes are a variation of VLPs. Currently they are generated using a two-step process: 1) detergent solubilization, which disrupts the viral envelop and allows removal of the nucleocapsid, followed by 2) extraction of the detergent, resulting in the spontaneous reassembly of the viral envelope along with the surface proteins. In the cases of influenza virus, the presence of HA and NA stabilize the integrity of the virosomes, as well as contribute to receptor-mediated endocytosis by the target cells [131,132]. Similar to classic VLPs, virosomes were first made to serve as simple vaccines but are now recognized to have many other potential purposes including the carriage of 'foreign' protein antigens [133] or other biologic payloads such as small interfering RNA [134] or drugs [135].

3. Immunostimulating Complex

Immunostimulating complexes (ISCOMs) are also considered to be VLPs in some respects. They are cage-like particles formed by mixing antigen, cholesterol, phospholipid, and Quil-A saponin in specific stoichiometric ratios

[136]. These complexes have been actively promoted as vaccine delivery systems due to their strong immune-stimulating properties. However, the requirement for the antigen to be hydrophobic limits the application of ISCOMs as antigen-delivering vehicles. ISCOMATRIX™ (Iscotec AB, Sweden) has the same general physical composition as ISCOMs, but without the antigen. Used as vaccine adjuvant, this formulation offers much broader application as it can be administered with both hydrophilic and hydrophobic antigens. Due to their ability to induce strong humoral and cellular immune responses, some ISCOMs and ISCOMATRIX™ vaccines have been approved for veterinary use, and several candidate vaccines are currently undergoing clinical trials for human use [136,137,138].

1.4.2. VLP-based Influenza Vaccines

With limitations exist among the current standard influenza vaccines (limited efficacy and length of production period), VLP becomes potentially an attractive alternative to the current inactivated and live-attenuated influenza vaccines. Its non-infective nature means it is safe to be administered without causing disease, while simultaneously inducing strong immune response toward the antigen. Several studies have successfully demonstrated the ability of influenza VLP to generate protective immunity in animal models such as mice and ferrets. In one study, intramuscular administration of 3µg of VLPs (cVLP containing HA, NA, and M1 proteins) based on the Fuji/02 H3N2 influenza strain into Balb/c mice elicited higher anti-HA antibody titers than those immunized

with whole inactivated viruses or recombinant HA. In this same study, ferrets given 15µg of cVLPs IM developed broad spectrum of antibodies that conferred cross-protection against antigenically distinct H3N2 isolates [139]. Similarly, Quan *et al.* studied the immunogenicity of influenza VLPs (cVLPs) based of A/California/04/2009 strain in female Balb/c mice. The VLPs contained both viral HA and M1 proteins were administered IM. Animals that received 0.1µg of VLPs (roughly 0.01µg of HA) were completely protected against lethal challenge with A/California/04/2009, and partially protected from A/PR/8/1934 challenge [140]. In another study, Song et al. constructed influenza VLPs that contained only HA proteins (cVLPs) using the sequences of A/Vietnam/1203/04 H5N1 influenza virus. Immunized mice intradermally (ID) with 2µg of VLP (roughly 0.2µg of HA proteins) not only produced high antibody titers (IgG1 and IgG2a), but the mice also survived the homologous viral challenge [141].

These studies show that influenza VLP vaccines can indeed provide protective immunity during the challenge in animal models. Furthermore, they answered some interesting questions regarding VLPs as vaccine candidate. First, it is possible to develop broad protective immunity from VLP constructs that only contain one type of protein [141]. Second, the particulate nature of VLP may contribute to the development of stronger immune response, as suggested by Bright et al.

1.4.3. Generating VLP-based Vaccines in Cell-based System

The systematic study of VLPs began with the observation of HBV-like particles in the sera of the HBV-infected patients (Dane particles) composed entirely of HBV surface antigen [142]. First animal and then human studies rapidly demonstrated the ability of these HBV-VLPs to induce protective immune response against HBV infection. In the mid-1980s, purified HBV-VLPs isolated from patients' sera were used as the first generation HBV vaccine [143].

Methods for VLP production have evolved greatly since the first HBV vaccines. Many groups have tried different cell-based expression systems including bacteria, yeast and insect cells as well as mammalian cell lines to generate various viral capsid proteins that spontaneous assemble into particles.

One popular method of VLP production is based upon the baculovirus/insect cell platform. In this method, the gene of interest is inserted into the baculovirus genome through homologous recombination. The virus carrying the gene of interest is then transfected into *Spodoptera frugiperda* Sf9 cells [144]. Under the influence of the viral promoter, the gene of interest is expressed at high levels within the cell, and the resulting protein is purified. For instance, Cervarix™ (GlaxoSmithKline) is a VLP-based HPV vaccine that was produced by introducing the L1 gene (encoding for one of the viral capsid proteins) into the baculovirus/Sf9 expression system. Once expressed, the L1 proteins self-assemble into empty capsid structure that is morphologically similar to real HPV virion [145].

Similarly, VLPs can also be produced using yeast as the expression system. The coding region of the gene of interest is cloned into the yeast expression vector driven by GAL10 promoter. *S. cerevisiae* (for example) is then transformed with the recombinant vector and high level expression of the target gene can be initiated and driven in the presence of YPD medium (contains yeast extract, peptone, and dextrose) [146]. This method is used to produce several VLP-based vaccines that are currently on the market, including the HPV vaccine licensed under the trade name Gardasil™ (Merck & Co.) and several of the HBV vaccines (e.g. Engerix-B™ from GlaxoSmithKline).

1.4.4. Evaluation of Cell-based Expression System

Descriptions for cell-based expression platforms for VLP productions are well established. However in cell-based systems, both capital investment and running costs (i.e. cost of goods) are expensive due to the need for industrial-size fermenters and the trained personnel [147]. This means that the cost per vaccine dosage increase and may restrict some populations from accessing the vaccines. These cell-based platforms also suffer in terms of scalability. In particular, cell-based platforms require good laboratory practice (GLP) incubation facilities that are in short supply around the world. There is limited "surge capacity" in fermenters, implying that this approach may not be able to produce sufficient numbers of vaccine doses in response to a sudden increase in market demand such as occurs in a pandemic.

1.4.5. Emergence of the Plant-based Expression Platform

Plant-based manufacturing has recently emerged as an attractive alternative to the cell-based systems. This platform is economically advantageous compared to cell-based systems from the business standpoint, as it can produce recombinant proteins at costs 90-98% below those of microbial fermenters, and 99.9% less than those using mammalian cell cultures [148]. This cost advantage per vaccine dose can make a significant difference in affordability for low- and middle-income countries. The scalability of the plant-based platforms gives it another commercial advantage over cell-base platforms [147,148,149]. While the expansion of production based on fermentation or tissue culture requires much more expensive equipment and materials, plant-based production can respond to increasing market demand by adding relatively low-technology and inexpensive greenhouse space [147].

1.4.6. Vaccine Production in Plant: Stable versus Transient Expression Systems

Vaccine antigen production using plant expression platforms is not a new concept. In fact, several attempts to construct stable transgenic vegetables that expressed vaccine antigens have been reported. Some of these transgenic vegetables were designed to be administered orally to elicit mucosal immunity. For instance, oral administration of dry transgenic tomatoes expressing synthetic DPT peptide (5.5 Lf unit of *diphtheria* toxoid; 73 ng of *pertussis* toxoid; 23.8 Lf unit of *tetanus* toxoid) in Balb/c mice elicited higher toxin-specific IgA secretion in the gut compared to control animals [150]. In another study, Balb/c mice fed

with transgenic potato leaf, expressing both fusion and hemagglutinin-neuraminidase proteins of Newcastle disease virus five times a week over a month, developed strong serum IgG1 and mucosal IgA responses to the viral antigens [151].

The use of transgenic plant to produce vaccine antigens presents certain challenges. First, the time required to verify successful transformation and achieve optimal protein expression can be a lengthy process (many months if not years). Using plants as the transgenic platform typically requires following the stable integration of the target gene for several generations before the confirming correct protein expression and later mass production. As a result, this approach is not particularly suitable for the production of influenza vaccines, where the antigens needed change from year to year and where rapidity is essential in the case of a pandemic. The use of transgenic plants to develop vaccines would also fall afoul of many people's (and even government's) sensibilities about what is 'safe' and what is 'not safe'.

Transient expression of target genes in plants overcomes many of the challenges associated with stable transformation. Transient expression systems were first developed to test and evaluate recombinant proteins on a small scale before committing to the expense and complexities of producing a transgenic plant [149]. This method does not require the integration of the target gene into the host genome before protein can be expressed, thus significantly shortening the

time required for the antigen production. These transient expression systems were later modified so that large quantities of proteins could be expressed and purified in very short periods of time (weeks).

1.4.7. Influenza VLP Production in Plant

Any safe and effective vaccine is a good vaccine. However, for influenza vaccines, one must also take cost and scalability into consideration due to the need for annual boosters caused by antigenic drift and the ever-present threat of a pandemic as the result of antigenic shift. In the last decade, the use of close relative of the tobacco plant, *N. benthamiana*, as a transient expression system has gain popularity among the vaccine community. Many groups have successfully produced different vaccine candidates that are capable of eliciting antigen-specific antibody response [152,153,154,155]. One of the groups, Medicago Inc. (Ste Foy, QC) has used the very same system, to produce influenza VLPs as candidate vaccines. In this platform, two groups of gram-negative bacteria, *Agrobacterium tumefaciens,* that contain different plasmids, are used as inoculant for the plants: one contains the plasmid encoding the gene of interest (influenza HA), and the second one contains the plasmid encoding the suppressor of silencing gene (its expression suppresses the plants' defense mechanism against bacterial infection). Agrobacteria are subsequently introduced into the extracellular space of plant leaves by agroinfiltration. The plasmids containing the gene-of-interest, as well as the suppressor gene are passed onto the plant cells through horizontal gene transfer. Large numbers of HA-VLPs are generated within 6-8 days of

agaroinfiltration which can then be easily purified from the plant biomass using sequential digestion and centrifugation. This was one of the first demonstrations of the production of enveloped VLPs bearing influenza antigens using plants as the transient expression platform.

1.4.8. Physical Characteristics of Plant-derived VLPs

Wide ranges of methods have been applied to study the morphology, structure, and composition of Medicago's plant-derived influenza VLPs after purification. Size-exclusion chromatography indicates that the VLPs are high-molecular weight structures. Electron microscopy analysis shows the particles to be around 100nm, with multiple copies of influenza HA present on the surface (**Figure 4**). Analysis of the lipid composition shows that the particles originate from the plant cell membrane and that the particles contain very little plant-origin protein. These observations confirm that influenza VLPs bud from the plant plasma membrane and the host proteins have largely been excluded from the membranes during the process [152].

1.4.9. Immunogenicity of Plant-derived VLPs

In 2008, Medicago Inc. and the McGill Vaccine Centre first studied the immunogenicity of plant-produced VLP in Balb/c mice [152]. For this candidate vacine, the genetic sequence of the H5 hemagglutinin antigen of the highly virulent avian H5N1 isolate (A/Indonesia/5/05) was cloned into a plasmid and introduced into *Nicotiana* plants for H5-VLP production. Several earlier studies

using various vaccine formulations [151,156] had shown that the H5 protein was a relatively weak humoral immunogen compared to other influenza HA proteins. In contrast, immunizing Balb/c mice with only 1 μg of the plant-based H5-VLP (two-dose schedule) elicited surprisingly strong antibody responses. Most importantly, injecting as little as 0.5 μg H5-VLP with or without alum was found to induce cross-clade protection in ferrets (eg: A/Indonesia/5/05 vaccination followed by A/Vietnam/1194/04 challenge) [157]. In addition to animal testing, this candidate H5-VLP vaccine is currently undergoing active clinical evaluation. The outcome from the phase I trial (unpublished data) suggests that this candidate H5 vaccine is well-tolerated and can elicit humoral responses comparable to or better that competitor vaccines at modest doses (data not shown; personal communication N. Landry, Medicago Inc).

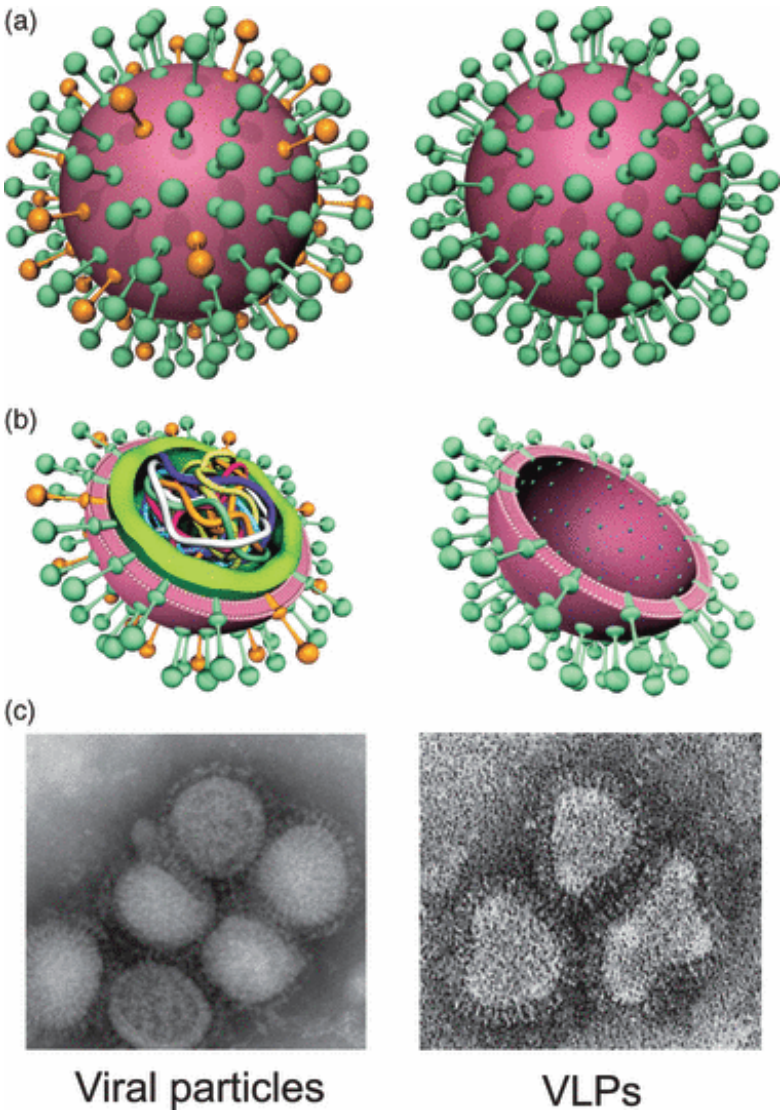

Figure 4: Schematic Comparison between Influenza Virus and Medicago's Virus-like Particles (VLPs). (Adapted from [158]). (a) AT the surface, both influenza virus and VLPs have viral protein (HA) embedded in their lipid bilayers. (b) Cross-sections showing the lack of viral capsid and viral genome as features of VLPs. (c) By electronic microscopy both viral particles and VLPs have similar morphology.

1.5. RATIONALE AND STUDY OBJECTIVE

Plant-derived VLPs represent a completely new tool in vaccinology. To date, most of the immunological studies with plant-derived VLPs have been done in animal models and relatively little is known about the effect of these VLPs in humans. Thus, the overall goal of this work is to better understand the mechanism(s) of plant-derived VLP immunogenicity using human peripheral blood mononuclear cells (PBMCs) as the study model. In this study, isolated human peripheral blood mononuclear cells (PBMCs) from healthy adult donors were used to characterize the innate immune response to plant-derived influenza H5-VLP *ex vivo*. We selected H5-VLP generated from the A/Indonesia/05/05 sequence for these initial studies due to the lack of prior immunity in the general public. The characterization of innate immunity to the H5-bearing VLPs was achieved by assessing proliferative responses, activation status, and cytokine profiles. The proliferative response of PBMCs was measured in two ways: tritiated thymidine incorporation and intracellular Ki67 staining by flow cytometry. CD69 expression (an indication of cellular activation) was measured by flow cytometry. Last, the level of cytokines produced by PBMCs in response to H5-VLP stimulation was measured by multiplex ELISA (Quansys), as well as by flow cytometric intracellular cytokine staining.

CHAPTER 2: MANUSCRIPT

Characterization of Innate Immune Response to *Nicotiana benthamiana*-derived Influenza H5 Virus-like Particles

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2.1. INTRODUCTION

The re-emergence of influenza virus variants is a serious global health concern. Each year, influenza viruses cause immense economic losses and also cause significant morbidity and mortality in humans [159,160]. In 2009, a new strain of influenza virus, porcine H1N1 (pH1N1), emerged and became a threat to the population. It was speculated that the substantial spread of pH1N1 was caused by a combination of 1) the absence of cross-protective immunity generated from the previous influenza vaccine strains in the population, 2) exposure to circulating H1N1 strain [100, 101], and 3) the delayed production of an appropriate vaccine.

The current method of influenza vaccine production relies on growing the viruses in embryonated chicken eggs. The vaccines are composed of detergent-split viral subunits, inactivated whole virion, or live-attenuated virus, which are either adjuvanted or non-adjuvated. A recent meta-analysis examined the immunogenicity of the influenza vaccine against pH1N1 and showed a seroconversion rate of >70% in subjects receiving only 1 dose of split vaccine [161]. Furthermore, the same analysis also showed that vaccines with an oil-in-water adjuvant are more immunogenic than non-adjuvanted or alum-adjuvanted vaccines [161].

Despite the reported efficacy of vaccines against their cognate virus, there are two general limitations regarding current influenza vaccines. First,

they often fall short in providing a broad spectrum of cross-protective immunity against new influenza strains that have undergone antigenic shift. This provides an explanation as to how the pH1N1 virus caused the pandemic. Second, using embryonated chicken eggs as the source of vaccine antigen production raises concerns about the amount of time require for vaccine production. The time from choosing a viral strain for vaccine production to the widespread distribution of the vaccine to the general population typically takes up to 6 months [104]. This period before a vaccine becomes available provides sufficient time for the virus to spread. This delayed production of the traditional influenza vaccines and the constant threat of emerging viruses prompt the need to develop novel vaccine platforms that can generate large quantities of vaccine doses in the shortest time possible.

New vaccines that confer cross-protection, as well as possessing shorter production time frames were proposed. Among many, the use of non-replicating virus-like particles (VLPs) represents an attractive alternative to the current egg-based influenza vaccine [139,140]. Recently, Medicago Inc. (Ste Foy, QC) introduced a platform that uses the tobacco plant *Nicotiana benthamiana* to produce influenza virus-like particles (VLPs) as candidate vaccines [152]. In this system, two groups of *Agrobacterium tumefaciens* that carry either plasmids containing the gene of interest (influenza HA) or a gene that suppress plant defense mechanisms are used as the inoculant for the plant. *Agrobacterium* are

introduced into the extracellular space of plant leaves by agroinfiltration. Then, the plasmids are transferred from *Agrobacterium* into plant cells through horizontal transfer. Large quantities of HA-VLPs are produced within one week of agroinfiltration and are purified from plant biomass using sequential digestion and centrifugation techniques. This system provides several advantages over the traditional egg-based platform. Firstly, the production of VLPs requires only the targeted genetic sequence and does not involve the handling of live virus. Secondly, the system demonstrates remarkable scalability and is able to produce large quantities of vaccine within one month of obtaining the sequencing of the pandemic strain [158]. Lastly, immunogenicity study using H5-VLP constructs showed that VLP vaccines were able to induce both high antibody titres and cross-protective immunity in immunized animals [152].

It is clear that plant-derived VLPs represent a new tool in vaccinology. To date, most of the immunological studies with plant-derived VLPs have been done in animal models and relatively little is known about the effect of these VLPs in humans. Thus, the overall goal of this work is to better understand the mechanism(s) of plant-derived VLP immunogenicity using human peripheral blood mononuclear cells (PBMCs) as the study model. In this study, isolated PBMCs from healthy adult donors were used to characterize the innate immune response to plant-derived influenza H5-VLP *ex vivo*. We selected H5-VLP generated from the A/Indonesia/05/05 sequence for these studies due to the lack of prior immunity in the general public. The characterization of innate immunity

to the H5-bearing VLPs was achieved by assessing proliferative responses, activation status, and cytokine profiles.

2.2. MATERIALS AND METHODS

2.2.1. Isolating Peripheral Blood Mononuclear Cells (PBMC)

Healthy adult donors (ages 18 years or older) were recruited from the McGill Vaccine Evaluation Centre and peripheral blood samples were collected for PBMC isolation. In brief, EDTA-containing blood was centrifuged at 300g for 10 minutes at 20°C. Plasma was removed and stored in -20°C freezer for further treatment. The remaining blood cells were diluted 2-fold in Hanks Balanced Salt Solution (HBSS; supplemented with magnesium and calcium) at room temperature (RT) (Wisent, St-Bruno, QC) before layering onto Ficoll-Hypaque (Lymphocyte Separation Medium, Wisent) for differential density gradient centrifugation at 1020g for 30 minutes at 20°C. PBMC were collected from the interface layer between HBSS and Ficoll-Hypaque medium. The cells were washed at least 3 times (300g for 10 minutes at 20°C) before resuspension at $2x10^6$ cells/mL in RPMI-1640 complete medium supplemented with 10% heat-inactivated autologous plasma, 10mM of HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*, Wisent), and 20µM of gentamicin (Wisent). PBMC were counted using a hemocytometer and viability was assessed using Trypan-Blue exclusion.

2.2.2. Ethical Approval

The protocol of sample collection from humans was approved by Biomedical D Research Ethics Board of McGill University Health Center. A

written, informed consent form was given to each donor prior to the blood collection.

2.2.3. Monoclonal Antibodies and Flow Cytometry Reagent

The following monoclonal antibodies were purchased from eBioscience (San Diego, CA): anti-IL-1² PE (CRM56), anti-IL-6 FITC (MQ2-13A5), anti-IL-8 APC (8CH), anti-TNF-± eFluor450 (MAb11), anti-Ki67 PerCP eFluor710 (20Raj1), anti-CD3 FITC (UCHT-1), anti-CD14 PerCP Cy5.5 (61D3), anti-CD14 APC eFluor780 (61D3), anti-CD19 PE Cy7 (HIB19), anti-CD19 APC (HIB19), anti-CD69 FITC (FN50), anti-HLA-DR PE Cy7 (LN3). The following monoclonal antibodies were purchased from BD Biosciences (San Jose, CA): anti-CD3 V500 (UCHT-1), anti-CD19 V500 (HIB19), and anti-CD56 PE Cy7 (B159). During staining, cells were washed with 1X PBS (Wisent). FoxP3 Fixation/Permeabilization Solution for nuclear Ki67 staining was purchased from eBioscience. Fixation and Permeabilization Solution, and Perm/Wash Buffer for intracellular cytokine staining were both purchased from BD biosciences. Non-viable cells were excluded using Fixable Viability Dye eFluor 780 from eBioscience. Brefeldin A, which was used to stop cytokine export, was purchased from eBioscience.

2.2.4. Lymphocyte Proliferation Assay by Tritiated Thymidine Incorporation

To measure proliferative responses, PBMCs were resuspended at a concentration of $2x10^6$ cells/mL in complete RPMI. 100µL of PBMCs were plated

in triplicate onto 96-well, polystyrene, round-bottom plates ($2x10^5$ cells/well) in the presence of the following stimuli: 10% complete RPMI medium only (negative control); various concentrations of H5-VLP ranging from 0.625 to 28µg/mL (provided by Medicago Inc., Ste-Foy, QC); recombinant H5 at 5µg/mL (Immune Technology, New York, NY); phytohemagglutinin (PHA) at a concentration of 2µg/mL (Sigma Aldrich). Cultures were incubated at 37°C in 5% humidified CO_2 incubator for total of 72 hours, with the addition of tritiated thymidine at 1µCi/well at 54 hours. Cellular DNA was harvested onto glass fiber filters using a 96-well harvester (Tomtec, Hamden, CT). The amount of tritiated thymidine incorporation was measured by the beta Liquid Scintillation Fluid Counter (GMI Inc. Ramsey, MN). Results are expressed as stimulation indices (S.I.), defined as the ratio of scintillation counts-per-minute (cpm) of antigen-stimulated wells and RPMI-only background control wells.

2.2.5. Measuring PBMC Proliferation with Ki67 Staining by Flow Cytometry

To identify the PBMC subsets that proliferate after co-culture with H5-VLP, PBMCs were stained with both cell lineage-specific antibodies: anti-CD3 FITC, anti-CD14 APC-eF780, anti-CD19 PE-Cy7, and anti-CD56 APC; and a marker associated with proliferation: anti-Ki67 PerCP-Cy5.5. Ki67 expression was measured by flow cytometry and the results were expressed as the percentage of Ki67-expressing cells in each PBMC subset. Briefly, PBMCs were resuspended at a concentration of $1x10^6$ cells/mL of 10% complete RPMI media, and co-cultured under the following conditions for 48 hours: 10% complete RPMI

media alone (Wisent), H5-VLP (2µg/mL, Medicago), recombinant H5 (2µg/mL, Immune Technology), or PHA control (1µg/mL, Sigma Aldrich). Cells were washed twice in cold PBS (Wisent) and centrifuged at 300g. After decanting the supernatant, 100µL of the subset antibody cocktail (combination of anti-CD3: 2µL/10^6 cells; anti-CD14: 1µL/10^6 cells; anti-CD19: 2µL/10^6 cells; and anti-CD56: 2µL/10^6 cells antibodies reconstituted in PBS) was added to each sample and incubated in the dark for 20 minutes. After incubation, cells were washed three times with cold PBS before fixation with 200µL of FoxP3 Fixation/Permeabilization Buffer (eBioscience) for 20 minutes. Fixed cells were washed twice with 1x Permeabilization Buffer (eBioscience), followed by addition of 100µl of anti-Ki67 (5µL/10^6 cells; reconstituted in 1x Permeabilization Buffer). Cells were incubated in the dark for 20 minutes before washing three times with 1x Permeabilization Buffer. After last wash, cells were resuspended in cold PBS. 500,000 events were collected to measure the expression of Ki67 using FACSCanto II (BD, San Jose). The results were analyzed using FlowJo software (Treestar, Ashland).

2.2.6. Cytokine Profiling by Multiplex ELISA (Quansys)

To further characterize the PBMC response to H5-VLP, a 16-plex ELISA-based array (Quansys, Loga, Utah) was used to analyze the cytokine profiles in culture supernatants collected at various time points after stimulation (6, 24, and 48 hours). This multiplex assay allows detection of: IL-1±, IL-1², IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-23, IFN-³, TNF-±, and

TNF-[2]. When necessary, supernatants were diluted with 10% complete 1640 RPMI media to increase the range of detection. All procedures were performed according to the manufacturer's instructions. The concentrations of the cytokines were calculated from standard curves included in each run.

Briefly, 50µL of each sample (in duplicates: undiluted and 1:2 diluted) was transferred to the multi-plex ELISA plates. The samples were incubated for 1 hour on the plate shaker at room temperature. Plates were then washed three times with 200µL/well of the supplied wash buffer; 50µL/well of the detection antibody was added to the plates and incubated for 1 hour at room temperature on an automatic plate shaker. Plates were then washed three times with wash buffer (as above) and 50µL/well of the Streptavidin HRP 1x reagent was added followed by a final 15-minute incubation at room temperature. Plates were washed six times with wash buffer before addition of 50µL/well of the chemiluminescence substrate. Samples were immediately analyzed by Quansys Q-View™ Imager, with software "Q-Image™" at 1-minute intervals. All reagents, ELISA plates, imaging system and the software were purchased from Quansys Bioscience.

2.2.7. Flow Cytometric Analysis of PBMC Activation

The state of PBMC activation after VLP stimulation was analyzed by measuring up-regulation of the cell-surface marker, CD69. PBMCs were resuspended at a concentration of 1 $x10^6$ cells/mL using 10% complete 1640-RPMI medium. Aliquots of PBMCs were placed into 15mL conical tubes, each tube representing a different condition: negative control 10% complete RPMI

media, H5-VLP (5µg/mL), recombinant H5 protein (5µg/mL), and positive control PHA (5µg/mL; Sigma Aldrich). Tubes were incubated in the 37°C, CO_2 chamber for 6, 24, or 48 hours prior to staining. 500,000 events were collected using BD FACSCanto II (San Jose, CA), and the results were analyzed using FlowJo software (Tree Star, Ashland, OR).

2.2.8. Intracellular Cytokine Staining (ICS) by Flow Cytometry

PBMCs were transferred to 96-well U-bottom tissue-culture plates (BD Biosciences) in complete media at a final concentration of $5x10^5$ cells/100µL. Each well received one of the following treatments: 10% complete RPMI media alone (Wisent), H5-VLPs (5µg/mL, Medicago), or recombinant H5 (5µg/mL, Immune Technology), or PHA (2 µg/mL, Sigma Aldrich). Cultures were kept at 37° C in a 5% CO_2 humidified incubator for the indicated length of time (6, 24 or 48 hours). Three hours prior to the end of incubation, 1x brefeldin A (eBioscience) was added to inhibit cytokine release by Golgi blockade. Our intracellular cytokine staining protocol was adopted and modified from Lamoreaux et al [162]. Briefly, PBMC were centrifuged at 400*g* for 5 minutes at 4°C, washed twice with PBS and stained with Fixable Viability Dye eFluor780 (eBioscience) for 15 minutes. PBMCs were then washed twice with cold PBS (Wisent) with 1% BSA (Sigma Aldrich). PBMCs were stained for 15 minutes on ice with anti-CD3, anti-CD56 (BD Horizon, BD Pharminogen, respectively, San Jose, CA), anti-CD14, anti-CD19, and anti-HLA-DR (eBioscience) antibodies. Surface-stained PBMC were washed with cold PBS, and then fixed and permeabilized for 20 minutes with Cytofix/Cytoperm solution (BD Biosciences). PBMCs were washed twice with 1x Permeabilizing Buffer (BD

Biosciences) to remove residual fixation buffer. The intracellular-staining antibody cocktail consisting of anti-IL-1², anti-IL-6, anti-IL-8, and anti-TNF-± (all at concentration of 2µL/10^6 cells; eBioscience) was added to PBMCs. PBMCs were stained for 20 minutes on ice in the dark before washing twice with 1x Permeabilizing buffer solution and resuspension in 100µL of PBS solution. All samples were run on an 8-color FACSCanto II cytometer with BD FACS DIVA software. 500,000 events were collected and analyzed using FlowJo analytical software (Tree Star).

2.2.9. Agrobacteria LPS Neutralization

Human PBMCs were resuspended in 10% complete RPMI media at a concentration of 5x10^6 cells/mL. 100uL of the cell suspension was added to individual wells in 96-well round-bottom plates with the following reagents with or without 20µg of polymyxin B (PMB; Sigma Aldrich): 10% complete RPMI media, H5-VLPs (5µg/mL), recombinant H5 proteins (5µg/mL), or *E. coli*-derived LPS (0.34EU; Sigma.Aldrich) Cells were cultured at 37°C in a humidified, CO_2 incubator for 6 hours with 1x brefeldin A added for the last 3 hours. At the end of incubation, cells were washed twice with 200µL of PBS (with 1% BSA; Wisent) and stained with surface marker anti-CD14 PerCP-Cy5.5 (eBioscience) at concentration of 1µL/10^6 cells on ice and shielded from light source for 20 minutes. After the surface staining, cells were washed twice with 200µL of cold PBS before the addition of 200µL of Cytofix/Cytoperm buffer (BD Biosciences) on ice, in the dark for 20 minutes. PBMCs were washed twice with 1x Permeabilization buffer (eBioscience) to remove the residual fixation buffer.

Intracellular cytokine staining antibody cocktail consists of anti-IL-1² PE (2µL/10^6 cells; eBioscience), and anti-TNF-± eFluor450 (2µL/10^6 cells; eBioscience) were reconstitute in 1x Permeabilization buffer solution to the final volume of 100µL before adding to each well. PBMCs were stained for additional 20 minutes on ice and shielded from light source. The cells were washed twice with 1x Permeabilization buffer solution before resuspended in 100µL of PBS solution. 500,000 events were collected to measure the expression of IL-1² and TNF-± in $CD14^+$ cells using FACSCanto II (BD Biosciences), and the results were analyzed using FlowJo (Treestar).

2.3. EXPERIMENTAL RESULTS

2.3.1. Human PBMCs Proliferate In Response To Influenza H5-VLP

To investigate whether human PBMCs (PBMCs) are capable of responding to plant-made influenza H5-VLP, we measured the proliferative response of PBMCs by the tritiated thymidine incorporation assay. PBMCs were cultured in 10% complete RPMI, with four concentrations of H5-VLP (1.25, 2.5, 5, and 10μg/mL), or phytohemagglutinin (PHA; 2.5μg/mL) as a positive control for 72 hours. At the lowest concentration tested, H5-VLP (1.25μg/mL) induced a significant proliferative response in PBMCs (**Figure 5A**; mean ± SEM S.I. = 4.0 ± 0.9; n=8, $P<0.05$). The S.I. appeared to increase steadily at higher concentrations of H5-VLP (**Figure 5A**) but the differences did not reach statistical significance.

To determine whether this effect was a unique characteristic of the H5-VLP, or a general feature of the H5 protein itself, proliferation we included 5μg/mL of soluble recombinant H5 protein (rec H5) as a control. While the rec H5 protein elicited a detectable proliferative response (**Figure 5B**; mean ± SEM S.I. = 3.1±0.8; n=8), the level of proliferation was significantly lower than that observed in cultures exposed to the same concentration of H5-VLP (**Figure 5B**; mean ± SEM S.I. = 6.3 ± 1.2; n=8; $P<0.05$).

2.3.2. CD19+ B Cells and CD3+/CD56+ NKT Cells Up-regulate Expression of Ki67 After Co-cultured With H5-VLP

One limitation of using thymidine incorporation to measure cellular proliferation is the lack of information on the specific cell-type responding. To identify the PBMC subsets actively entering cell cycle in response to H5-VLP, PMBCs were cultured in 10% complete RPMI media with H5-VLP (2μg/mL), rec H5 protein (2μg/mL), or PHA (1μg/mL) for 48 hours, and the percentage of individual PBMC subsets expressing the proliferation marker, Ki67, were analyzed by flow cytometry. Using multi-parameter flow cytometry, we targeted five PBMC subsets for analysis: $CD14^+$ cells (monocytes, dendritic cells), $CD3^-$ /$CD56^+$ NK cells, $CD3^+$/$CD56^+$ NKT cells, $CD3^+$/$CD56^-$ T cells, and $CD19^+$ B cells (**Figure 6A**). There was no change in the percentage of Ki67 expressing $CD14^+$ cells (data not shown) or the $CD3^+$/$CD56^-$ T cells after stimulation with H5-VLP or rec H5 (**Figure 6B**; n=4; $P>0.05$). We observed a higher percentage of Ki67 expressing $CD3^-$/$CD56^+$ NK cells after incubation with H5-VLP in comparison to either media or rec H5 protein; however, this increase did not reach statistical significance (**Figure 6D**; n=4; $P>0.05$). In contrast, there were significant increases in the percentage of both Ki67 expressing $CD19^+$ B cells and $CD3^+$/$CD56^+$ NKT cells after stimulation with H5-VLP (**Figure 6C and E**; n=4; $P<0.05$, $P<0.01$ respectively), but not rec H5.

2.3.3. CD69 Expression Was Up-regulated In CD3+/CD56- *T Cells, CD19+ B Cells and CD3-/CD56+ NK Cells In Response to H5-VLPs*

The up-regulated expression of CD69 on the plasma membrane is a common marker of activation in a wide range of immune cells, particularly during the early stages of the immune response. To assess immune cell activation, we used flow cytometry to measure the percentage of CD69 expressing CD3+/CD56- T, CD19+ B, CD3-/CD56+ NK, and CD14+ cells after 6 hour co-culture with 10% complete RPMI alone, H5-VLP (5μg/mL), or rec H5 protein (5μg/mL). At 6 hours, we observed a slightly increase in CD69-expressing CD3+/CD56- T cells in response to H5-VLP compared to control cell cultures (**Figure 7A**; n=9; $P<0.05$) and cultures stimulated with rec H5 proteins. CD69 expression was also increased on CD19+ B cells after exposure to both H5-VLP and rec H5 proteins (**Figure 7B**; n=10; both $P<0.001$). In fact, the rec H5 induced significantly more CD69 expression on B cells than H5-VLP ($P<0.05$). Similar to the B cell response, both H5-VLP and rec H5 increased CD69 expression on CD3-/CD56+ NK cells (**Figure 7C**; n=9; both $P<0.001$ vs. media). In the case of the NK cells however, there was no significant difference in CD69 expression between H5-VLP and rec H5 stimulation. Finally, CD69 expression in CD14+ cells did not change in response to either H5-VLP or rec H5 (**Figure 7D**; n=7; $P>0.05$).

2.3.4. H5-VLPs Induce Secretion of Inflammatory Cytokines by PBMC

To further characterize the innate immune response to H5-VLP, a multiplex, ELISA-based, cytokine detection array was used to measure cytokine levels in the supernatants PBMCs cultured in 10% complete RPMI media and stimulated with H5-VLP (5µg/mL), rec H5 (5µg/mL), or PHA (2.5µg/mL) for 6 hours. The most striking changes were observed for the pro-inflammatory cytokines, IL-1², IL-6 and TNF-±. IL-1² concentration was 15-fold higher in the supernatant of PBMCs treated with H5-VLP than with media control (**Figure 8A**; mean ± SEM = 427.0±130.9 vs. 27.2±2.6 pg/mL, respectively; n=10; $P<0.001$). There was a 42-fold increase in IL-6 concentration with H5-VLP (**Figure 8B**; mean ± SEM = 1278±268.4 vs. media 30.4±4.7 pg/mL; n=10; $P< 0.001$) and a 10-fold higher concentration of TNF-± (**Figure 8D**; mean ± SEM = 436.3±83.1 vs. media 43.3±5.6; n=10; $P<0.001$). There were no significant increases in any other cytokine concentration measured at 6 hours post-stimulation with H5-VLP including IL-8 (**Figure 8C**; mean ± SEM = 731.7±62.6 vs. 1109±163 pg/mL; n=10; $P>0.05$), IL-2, IL-4, IL-5, IL-12p70, IL-17 and IFN-³ (data not shown).

Very similar increases in pro-inflammatory cytokine production were observed in PBMC cultures stimulated by rec H5. Concentrations of IL-1², IL-6 and TNF-± were all significantly greater than the media control cultures at 6 hours (**Figure 8A to D**; mean ± SEM = 195.8±67.8, 790.3±190.3, and 1384±122 pg/mL respectively; all $P<0.001$ vs. media alone). Compared to stimulation with H5-VLP, rec H5 seemed to induce slightly less IL-1², IL-6 and TNF± production and

more IL-8 production but none of these differences reached statistical significance.

We followed the kinetics of IL-1² and TNF-± secretion by PBMCs at 24 and 48 hour post-incubation with H5-VLP. Although the culture supernatants of H5-VLP- stimulated PBMCs had higher concentrations of IL-1² and TNF-± than media control cultures throughout this time period (P<0.01), the pattern of pro-inflammatory cytokine production under the different stimulation conditions remained the same (**Figure 8E and F**).

2.3.5. CD14+ Monocytes are the predominant cell type responsible for pro-inflammatory cytokine production in response to H5-VLP

The cytokine profile obtained from the multiplex ELISA analysis suggested a strong innate pro-inflammatory response from PBMCs stimulated with H5-VLP. To determine the cellular source of these cytokines, PBMCs stimulated with H5-VLP (5µg/mL), rec H5 (5µg/mL) or media alone for 6 hours were stained with lineage-specific and cytokine-specific antibodies, and analyzed by flow cytometry. Stimulation with H5-VLP led to a significant increase in the percentage of IL-1² expressing CD14+ cells (**Figure 9A**; mean ± SEM = 85.3±4.0% vs. 7.6±2.9 with media respectively; n=16; P<0.001). More CD14+ cells also expressed IL-1² after stimulation with rec H5 (**Figure 9A**; mean ± SEM = 52.2±6.9%; n=14; P<0.001); but this change was significantly lower than the cells exposed to H5-VLP (P<0.001). A very similar pattern was observed in the percentage of IL-6 expressing CD14+ cells at 6 hours after stimulation with H5-

VLP (**Figure 9B** mean ± SEM = 44.8±6.0 vs. 0.3±0.1 with media; n=9; *P*<0.001) and rec H5 (mean ± SEM = 19.8±7.4%; n=7; *P*<0.05 vs. H5 VLP). Both H5-VLP and rec H5 induced more percentage of TNF-± expressing CD14+ cells than media control (**Figure 9D**; mean ± SEM = 7.2±1.2, 7.3±1.8 and 0.7±0.1, respectively; n=16; all *P*<0.001) with no difference between the H5-VLP and rec H5 stimulated cultures.

Although no significant differences in supernatant IL-8 were observed using the multiplex ELISA, there were nonetheless significant increases in the percentage of IL-8 expressing CD14+ cells in the H5-VLP stimulated cultures compared to media control (**Figure 9C**; mean ± SEM = 30.7±7.7 vs. 1.4±0.3%; n=16; *P*<0.001). Rec H5 protein also increased the percentage of IL-8 producing CD14+ cells above the media control value (**Figure 9C**; mean ± SEM = 22.3±5.8%; n=16) but the difference between the H5-VLP and rec H5 treated cells did not reach significance.

There were no significant differences in pro-inflammatory cytokine expression in any of the other PBMC subsets studied (CD19+ B cells; CD3-/CD56+ NK cells; and CD3+/CD56- T cells. **Figure 10-12**, respectively. n=4 to 13) at 6 hour after stimulation with media alone, H5-VLP, or rec H5.

In summary, our finding suggested that pro-inflammatory cytokines were mainly produced by CD14+ cells in response to either H5-VLP or rec H5.

2.3.6. Plant-derived H5-VLP stimulate pro-inflammatory cytokine production in CD14+ cells in the absence of Agrobacterium LPS

The use of a gram-negative bacterium, *Agrobacterium tumefaciens*, as the vector in the plant-based VLP-expression platform raises the possibility of *Agrobacerium* LPS (a.LPS) contamination in the final VLP preparation. To ensure the observed inflammatory cytokine responses were not caused by a.LPS in our H5-VLP preparation, 10% complete RPMI media, H5-VLP (5µg/mL), rec H5 (5µg/mL), and positive control *E. coli* LPS (e.LPS; 1.7 EU/mL; the concentration was chosen to match the concentration of a.LPS present in the lot of H5-VLP used in this experiment) were either pre-incubated with or without polymyxin B (PMB) before co-culturing with PBMCs for 3 hours. PMB is a chemical known to bind to and neutralize LPS. The levels of IL-1² and TNF-± in CD14+ cells were measured by flow cytometry and the results were expressed as histogram with median fluorescence intensity (MFI; **Figure 13 A-C**).

Our preliminary data showed that PMB itself had little or no impact on the expression of IL-1² in CD14+ cells at 3-hour post-stimulation (**Figure 13D**; IL-1² MFI in complete RPMI media pre-PMB vs. post-PMB: 485 vs. 275). Co-culturing with e.LPS stimulated IL-1² expression in CD14+ cells (MFI = 2555) but when e.LPS was pre-treated with PMB, the level of IL-1² returned to baseline levels (MFI = 318), confirming that PMB can neutralize the effect of e.LPS on CD14+ cells. Co-culturing CD14+ cells with untreated H5-VLP stimulated the expression of IL-1² (MFI = 3644). Pre-treatment of H5-VLP with PMB reduced IL-1² expression in CD14+ cells (MFI = 1422) but expression was still 5-fold

higher than the baseline level (**Figure 13D**). Stimulation with rec H5 protein also induced low level IL-1² expression in CD14+ cells (MFI = 794) but PMB-treatment reduced IL-1² expression to background levels (MFI = 256). This observation is surprising because the H5 recombinant protein was apparently expressed in 'LPS-free expression system' (human embryonic kidney 293 cell line, according to the manufacturer). The expression of TNF-± in CD14+ cells was also examined. Similar to IL-1², we observed elevated TNF-± expression in CD14+ cells in response to both e.LPS and H5-VLP compared to the media control (**Figure 13E**; MFI of complete RPMI media, e.LPS, H5-VLP = 368 vs. 1757 vs. 3348, respectively). As seen with IL-1², TNF-± expression in CD14+ cells dropped to the baseline level when stimulated with e.LPS pre-treated with PMB (MFI = 420). PBMC stimulated with H5-VLP pre-treated with PMB also had reduced TNF-± expression, but intracellular TNF-± levels still exceeded background levels (**Figure 13E**; MFI = 768). Expression of TNF-± in CD14+ cell in response to rec H5 remained at the baseline level with or without PMB pre-treatment (MFI of untreated vs. pre-treated PMB = 344 vs. 309).

These preliminary data suggest that contaminating a.LPS likely contributes to the pro-inflammatory cytokine production by PBMC in response to H5-VLPs. However, these data also suggest that plant-derived H5-VLP themselves can directly induce a proinflammatory cytokine response in human PBMC CD14+ subset.

2.4. GENERAL DISCUSSION

In this study, we examined the immune response to plant-derived H5-VLP. The subjects enrolled were presumably never to have been exposed to the target antigen (HA protein from A/Indonesia/20/05) because A/Indonesia is not endemic in North America. Thus, any response observed in the human immune cells was considered to be either innate or antigen non-specific in nature. Measures of what we believe to be innate immune responses to H5-VLP included cellular proliferation, expression of activation markers and cytokine production profiles in antigen naïve human PBMCs.

We observed that human PBMCs responded to H5-VLP stimulation with a significant increase in the amount of tritiated (H^3) thymidine incorporated, which is indicative of DNA synthesis, and by extension, cellular proliferation [163]. At lower doses (0.625-10μg/mL), the response to the H5-VLP in human PBMCs was dose-dependent, with higher levels of proliferation as the concentration of VLP used in the co-cultured increased. This was expected since an increase in VLP concentration likely strengthens the activation signals, thereby increasing the degree of proliferation. At higher concentrations of H5-VLP (>10μg/mL), we observed a decline in the degree of H^3-thymidine incorporation. We speculate that the decline in cellular proliferative response was due to hyperstimulation-induced cytotoxicity. To confirm the viability of the PBMCs at higher concentration, we performed trypan-blue staining after co-cultured. We noticed an increasing in the number of dead cells at high H5-VLP concentration (30μg/mL) compares to at

low dose (5μg/mL), suggesting lack of H^3 incorporation at high VLP concentration can be partially explained by cell death (data not shown).

We included recombinant H5 in our assays as an experimental control to determine whether proliferative responses to the H5-VLP were induced by the H5 protein or the VLP portion of the H5-VLP. Our data showed that the recombinant H5 protein also induced readily detectable proliferation in presumably naïve PBMC, albeit at a lower level than observed with H5-VLP stimulation. This result suggests that the proliferative response to H5-VLP is at least partially attributable to the H5 protein itself.

To determine which PBMC subpopulations were proliferating in response to the H5-VLP, we measured Ki67 expression, a cellular proliferation marker, after 48 hours of incubation with H5-VLP or recombinant H5. Since both B cells and NKT cells proliferated in response to H5-VLP stimulation but only B cells appeared to proliferate in response to the recombinant H5 protein, it seems likely that the H5 protein itself has mitogenic effect on B cells while the VLP portion of the H5-VLP accounts for the NKT cell proliferation. However, the B cell responses observed after recombinant H5 stimulation barely reached statistical significance (P=0.069, n=4). To clarify the involvement of H5 protein alone in B cell proliferation, these studies will need to be repeated with a larger number of samples.

The detection of CD19+ B cell proliferation was unexpected since B-cell expansion generally occurs much later in the immune response to a previously unknown antigen [164,165]. One possible explanation for the B cell proliferation

in response to the H5-VLP could be the very high density of H5 antigen on the VLP. The highly repetitive antigen display on the surface of the H5-VLP could potentially cross-link multiple B cell receptors (BCRs) to generate strong T cell-independent activation signals [166,167,168]. Such receptor cross-linking would be expected to transduce activation signal through kinase Bkt, ultimately leading to the maturation and expansion of B cells [169]. Depending upon the physical characteristics of the recombinant H5 antigen (i.e. fully soluble vs. aggregated) which was not assessed in these studies, this explanation might also account for the lower level of apparent B cell proliferation observed following recombinant H5 stimulation that did not reach statistical significance.

It was also surprising that the CD3+/CD56+ NKT cells expressed Ki67 after H5-VLP stimulation. NKT cells are very small subset of the total T cell population (between 0.1-1%) that can be activated upon recognition of lipid antigens presented in a CD1d-dependent manner [170,171]. Recent studies have highlighted the importance of NKT cells in the context of protection from highly pathogenic influenza virus infection [172,173]. Mice lacking NKT cells were unable to achieve viral clearance and subsequently succumbed [173,174]. NKT cells can also produce prodigious amounts of both Th1- and Th2-type cytokines under appropriate conditions and may play a central role in orchestrating the immune response to viral infection. Since NKT cells recognize lipid antigens and our VLPs are bound by plant plasma membrane rich in lipids, it is certainly plausible that plant-derived lipids contribute to the NKT cell proliferation we observed. Indeed, recent analysis on the VLP has revealed the presence of ±-

galactoceramide in the *N. benthamiana*-derived H5-VLPs (unpublished data). ±-galactoceramide is a glycolipid that is known to activate NKT cells [170,171]. It is likely that NKT cells proliferated as the result of stimulation from ±-galactoceramide of plant-derived VLPs. To confirm the involvement of ±-galactoceramide in NKT cell expansion, empty VLP (ie: plant-derived VLP without antigen construct) should be included in our *ex vivo* proliferation assay. Alternatively, immunize CD1d knockout or NKT cell-deficient mice with *N. benthamiana*-derived VLP, followed by either *in vitro* analysis of immune response or *in vivo* challenge study, may also elucidate the role of NKT cells in generating proper immune response to the antigen-bearing VLPs.

Finally, we did not observe an increase in Ki67 expression on CD14+ monocytes after H5-VLP stimulation. Human monocytes have long been thought to have no (or very limited) capacity to proliferate under normal circumstances [175]. However, recent evidence suggests that monocyte proliferation can occur when they are exposed to high concentrations of colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [176,177]. The absence of of CD14+ monocyte proliferation in our experiments could therefore be due to low levels of these cytokines in the culture supernatants. In future experiments, the concentrations of M-CSF and GM-CSF will be measured in the supernatants of H5-VLP stimulated cultures.

We examined the activation state of human PBMC subpopulations after 6 hours of stimulation with H5-VLP using flow cytometry, using CD69 as a general activation marker. CD69 is an early activation marker (peaked at 6-12 hours post stimulation) and is expressed by all PBMC subsets upon stimulation [178]. Our flow cytometric analysis on CD69 expression in PBMC subsets showed a small increase in the percentage of T cells expressing CD69 after H5-VLP stimulation, which could be the result of antigen recognition through T cell receptor. This is suggested by previous studies where naïve T cells were shown to increase CD69 expression within 24 hours of encounter with a novel antigen [179,180]. To confirm this possibility, the interaction between fluorescent-tagged VLPs and other immune cell in early time points should be investigated using fluorescent-based microscopy technique.

Similar to CD3+ T cells, we also noticed a moderate increase in CD69 expression on CD19+ B cells following stimulation with either H5-VLP or recombinant H5 protein. Again, this further demonstrates that H5 protein, as part of the VLP, may partially contribute to B cell activation. However, these observations also raise a question as to how B cells get activated by H5-VLP. In this study, we observed that 3-40% of the circulating human B cell population respond to H5-VLP stimulation by up-regulating surface CD69 expression; this large variation in percentage may be attributed to the difference in genetic background of individuals. There are at least two explanations for B cell activation in response to H5-VLP stimulation. First, the contaminants present in the H5-VLP preparation (*Agrobacterium* LPS; discussed more below) may play a

role in B cells activation during the stimulation with H5-VLP. Second, the activation of B cell may be caused by direct recognition of antigen through receptor binding. However, we do not think that H5-specific B cell receptors (BCR) are responsible for antigen recognition since the donors are expected to be naïve to H5 protein and therefore, the percentage of such a population is miniscule. In contrast, there is a possibility that glycoproteins on the surface of B cells contain ±-(2,3) linked sialic acid, which is a known receptor for avian H5 protein [181]. Therefore, we hypothesize that B cell activation is linked to the recognition of H5 protein through ±-(2,3) sialic acid on the cell surface. To confirm this hypothesis, the first step would be to identify the existence of ±-(2,3) sialic acid on the surface of B cells. After this verification, we can fluorescently tag both VLP and sialic acid, then using fluorescent microscopy, to determine whether they are indeed co-localize and induce B cell activation.

Although CD3-/CD56+ NK cells did not proliferate at 48 hours in response to either H5-VLP or recombinant H5 protein, we found strong CD69 expression on NK cells, suggesting that this cell population was also responding to these stimuli. Recent studies have demonstrated that functional activation of NK cells can be achieved through direct recognition of influenza hemagglutinin molecules including H5 by the NK cell receptors, NKp44 and NKp46 [182,183]. Both of these receptors belong to the family of natural cytotoxicity receptors (NCRs) that are characterized by their ability recognize viral antigen, leading to initiation of NK cell cytotoxic activity [184]. It has also been reported that mice lacking NKp46 receptors are much more susceptible to influenza infection, which

suggests the involvement of this receptor in viral clearance [185]. In our study, NK cells activation might be the result of receptor-ligand binding between NCRs (possibly NKp46) and H5 antigen. Since we observed NK cell activation following both H5-VLP and recombinant H5 stimulation, this process is likely independent of the physical composition of the VLP. To further our understanding of the NK cell role in shaping VLP-induced immune responses, we hope to conduct a thorough VLP-specific NK cell functional analysis (cytokine profile, or challenge study in NK-cell deficient mice).

Last, we did not observe significant change in CD69 expression on CD14+ cells in response to either H5-VLP or recombinant H5 stimulation. This observation could be due to the differential responses against various antigens by CD14+ cells. A previous study has shown that CD14+ cells respond to *E. coli* LPS stimulation by up-regulating CD69 expression, whereas such up-regulation is not seen when the cells are stimulated with muramyl dipeptide [186]. Studies using other activation markers such as anti-CD86 (an activation marker for antigen-presenting cells) or anti-CD40 antibodies (co-stimulatory molecules on antigen-presenting cells) [187] may provide a better evaluation of CD14+ cells activation state in response to H5-VLP than using anti-CD69 antibody.

To further characterize the innate response to H5-VLP, cytokine concentrations in the supernatants of VLP-stimulated PBMC cultures were determined by multiplex ELISA (Quansys). We found rapid and copious production of several pro-inflammatory cytokines (IL-1², IL-6, and TNF-±) in

response to H5-VLP stimulation. A similar pattern of response but at much lower intensity was also observed in recombinant H5-stimulated cultures. Given the intensity of the *in vitro* response to the H5-VLP, it is very interesting that none of the 36 subjects in Medicago's clinical trials of either H5 or H1 VLPs have reported anything more than mild local effects and rare and transient systemic adverse events ([157]; unpublished results). One possible explanation for this observation is that the microenvironment is very different between the *in vivo* 'system' (i.e. the subject's deltoid muscle) and the *ex vivo* 'system' (i.e. the 96-well plate). In fact, it stands to reason that the regulation of an "over-active" response would be much better *in vivo* than *in vitro*. Nevertheless, these findings lend further support to the notion that the non-H5 portion of the H5-VLPs may act as an intrinsic adjuvant in strengthening the immune response to the H5 antigen.

We also sought to determine the source of the pro-inflammatory cytokines in individual PBMC subset after exposure to H5-VLP and showed that most of the IL-1², IL-6, IL-8 and TNF-± produced in response to stimulation with H5-VLP was attributable to CD14+ cells. In the case of IL-8, we did not find higher concentrations of this cytokine in the H5-VLP stimulated cultures, but intracellular cytokine staining clearly revealed a response (**Figure 9C**). This observation could be due to the accumulation of IL-8 within the CD14+ cells before its release. CD14 is typically expressed by circulating monocytes [188], but the expression is not exclusive to these cells; macrophages [189], certain subset of dendritic cells [190], and even B cells [191], are also known to express

CD14. We believe that monocytes, but not other cell types, are the first responder to H5-VLP in our experiment setting for two reasons. First, dendritic cells and macrophages are mostly found in tissue, not in circulation [192,193]; and second, our gating strategy in flow cytometric analysis excludes B cells using B-cell specific antibody. Thus, circulating CD14+ monocytes are likely the PBMC subsets that produce these pro-inflammatory cytokines in response to H5-VLP stimulation.

The production of pro-inflammatory cytokines in response to H5-VLP by CD14+ monocytes may be important for the development of immune response for several reasons. First, IL-1² and TNF-± increase the expression of adhesion molecules on the surface of blood vessels and lymphoid organs, which allows circulating immune cells to dock and infiltrate the site of antigen presentation [194]. In addition, VLP- stimulated IL-8 production by monocytes could lead to the recruitment of neutrophils and other granulocytes which can phagocytose the target antigen and transport it to nearby draining lymph nodes for antigen presentation [195]. Furthermore, cytokines such as IL-6 can drive monocyte differentiation into macrophages [196]. Since macrophages are competent antigen presenting cells, the rapid expression of IL-6 in response to H5-VLP stimulation may accelerate the initiation of competent T and B cell responses to the H5 antigen. Taken together, the expression of pro-inflammatory cytokines by CD14+ cells following H5-VLP stimulation likely represent the first step in the development of the adaptive immune response to the target antigen.

Medicago's VLPs are produced in *N. bethamiana* (a close relative of the commercial tobacco plant) that are transfected via agroinfiltration with *A. tumefaciens* carrying the target antigen gene. *A. tumefaciens* is a Gram-negative bacterium that readily infects many plants and, like virtually all bacteria, has lipopolysaccharide (LPS) as a component of its cell wall [197]. As a result, the VLP produced by Medicago in plants may be contaminated by small amounts of bacterial LPS. Indeed, depending on the precise process used, the final content of presumed *A. tumefaciens* LPS in the VLP preparations varies from 0.28-2.8 EU/μg of H5 proteins (unpublished data). Although the LPS of *A. tumefaciens* is far less dangerous than LPS from other bacterial species [198], it is nonetheless an important variable to consider in our studies. To address the question of whether the inflammatory responses to plant-derived VLP were induced by LPS contamination, we pre-treated the VLPs with Polymyxin B (PMB). PMB is known to neutralize LPS and inhibit LPS-induced inflammatory cytokine production [199,200]. Consistent with previous findings, we observed a reduction in the production of inflammatory cytokines IL-1² and TNF-± in CD14+ cells stimulated with PMB-treated *E. coli* LPS. We further demonstrated that H5-VLP treated with PMB was still able to stimulate IL-1² and TNF-± expression in CD14+ monocytes. Therefore, the plant-derived H5-VLPs can induce inflammatory cytokine production in CD14+ monocytes, but *Agrobacterium* LPS contamination in the H5-VLP vaccine can also contribute to the cytokine production in these cells.

As mentioned earlier, we also detected a reduction of both IL-1² and TNF-± expression in CD14+ monocytes co-cultured with PMB-treated recombinant H5 protein. This finding suggests the presence of endotoxin in the recombinant H5 preparation despite the fact that the protein was reportedly produced using a "LPS-free" expression system. To verify whether the recombinant H5 protein was contaminated with LPS, several aliquots were analyzed using the photometric method (Pyrogene™ rFC Assay, Lonza). Trace amount were indeed present in the recombinant H5 preparations at a concentration range (0.88-2.4 EU/µg of H5 protein) comparable to the levels of *Agrobacterium* LPS in Medicago's H5-VLP lots. The induction of monocyte cytokine production by the recombinant H5 can therefore be attributed, at least in part, to the presence of LPS in the preparation.

Lastly, although we did not investigate the impact of *Agrobacterium* LPS on B cells, we suspect that B cell proliferation and activation in response to H5-VLP might also be partially induced by the LPS contamination in the VLP preparation. To confirm the involvement of *Agrobacterium* LPS of VLP in activating B cells, we hope to pre-treat H5-VLP with PMB and include it into our proliferation and activation assay.

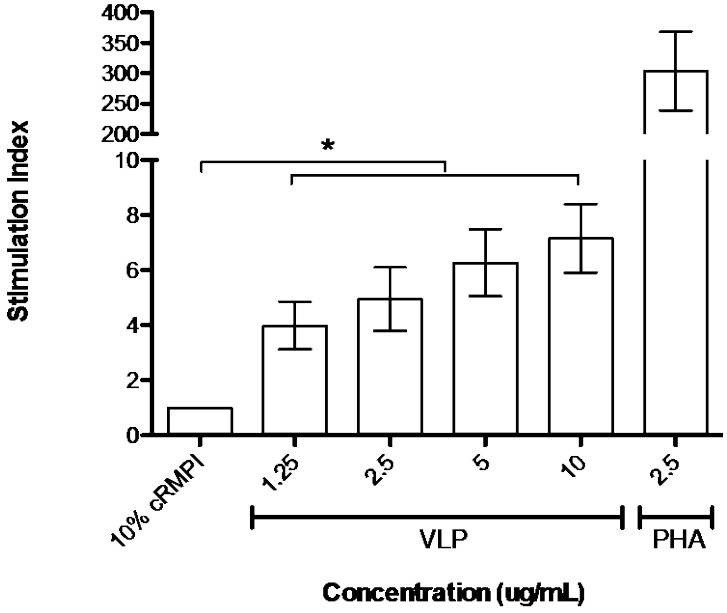
In conclusion, our work demonstrates that plant-derived VLPs bearing the HA molecule of the H5N1 influenza virus (A/Indonesia/05/2005) can elicit innate immune responses in PBMCs in H5-naïve individuals. Specifically, we show that these H5-VLPs induce cellular proliferation (primarily B and NKT cells), cellular activation (again, mostly B and NK cells) and the expression of pro-inflammatory

cytokines by CD14+ cells (presumably mostly monocytes). We further show that these effects are not driven by contamination of LPS. Our data, in combination with previous work, showing that the plant-derived VLP vaccines are safe and immunogenic in mice, ferrets [152] and humans [157], which provide strong support for the further development of these vaccine candidates. Compared to egg- and tissue culture-based technologies, the use of plants as a platform for vaccine production is cost-effective and can be easily scaled-up to meet the population requirements in the case of a pandemic [201]. Future work will focus on characterizing the mechanism of action of these novel vaccines, and ultimately, contribute to the design of other safe, inexpensive and effective VLP-based vaccine candidates.

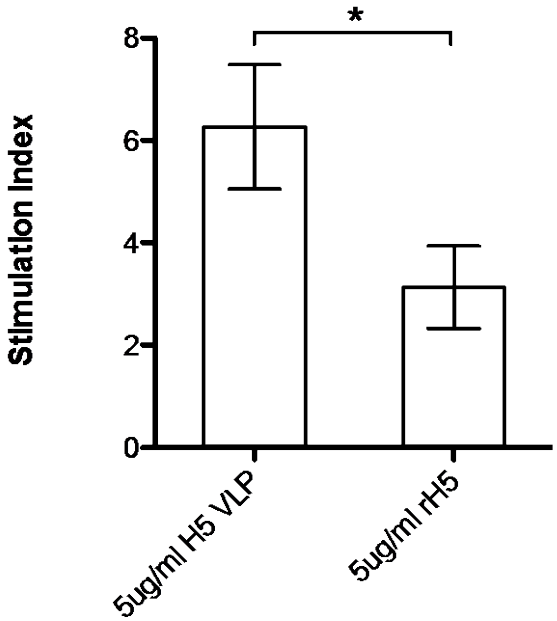
2.5. FIGURES

2.5.1. Figure 5: Human PBMC proliferation at 72 hours after co-culture with H5-VLP, measured by tritium uptake. Human PBMCs were isolated and co-cultured with complete RPMI media (10% cRPMI) or different concentrations of H5-VLP (1.25, 2.5, 5, and 10μg/mL) at a cellular concentration of $2x10^5$ cells/well. The cells were also co-cultured with recombinant H5 (5μg/mL), or PHA (2.5μg/mL) as controls. Proliferation was measured by the thymidine incorporation assay, and results are expressed as a stimulation index (S.I.; ratio of proliferation of stimulated cultures to unstimulated cultures). (A) PBMC stimulated with increasing concentrations of H5-VLP and harvested at 72 hours. The mitogen PHA was included as a positive control. (B) Comparison of the proliferative responses between PBMC stimulated with H5-VLP or recombinant H5 (both at 5μg/mL). Data represent means ± standard errors from 8 subjects. Statistical significance was determined by the Mann-Whitney test. *, $P<0.05$.

[A]

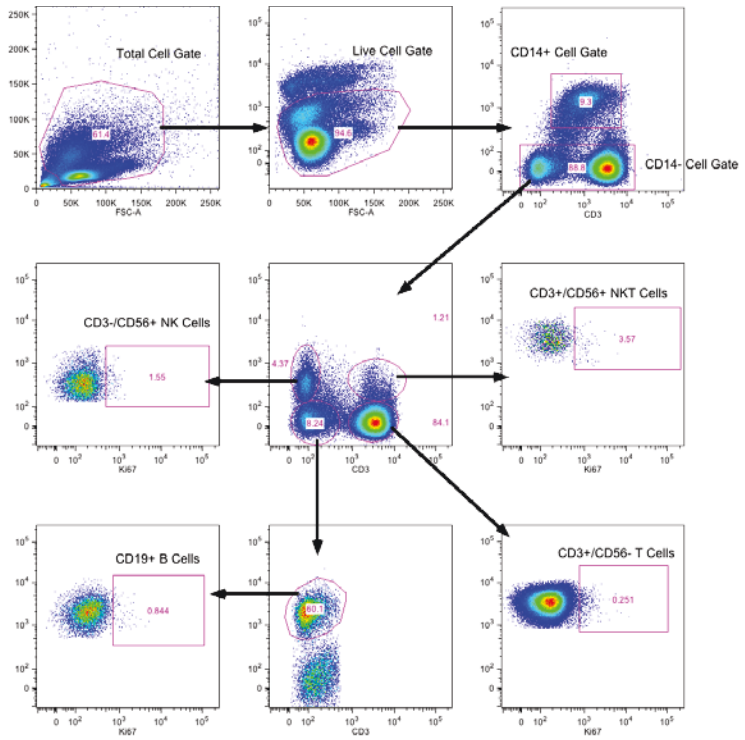

Stimulation Index
400
350
300
250
200
10
8
6
4
2
0
*
10% cRMPI
1.25
2.5
5
10
2.5
VLP
PHA
Concentration (ug/mL)

[B]

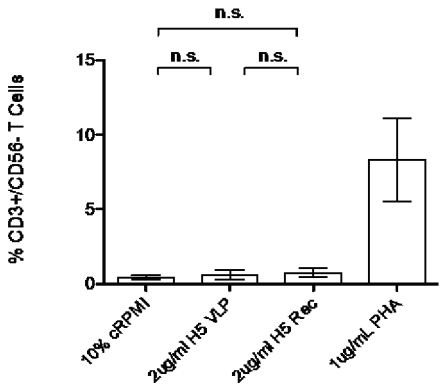

Stimulation Index
8
6
4
2
0
*
5ug/ml H5 VLP
5ug/ml rH5

2.5.2. Figure 6: Up-regulation of the proliferation marker, Ki67, was observed in CD19+ B cells and CD3+/CD56+ NKT cells co-cultured with H5-VLP for 48 hours. Human PBMCs were co-cultured with complete RPMI media (10% cRPMI), H5-VLP,, recombinant H5 (rH5; both at 2 μg/mL) or PHA (1 μg/mL) for 48 hours. Proliferation of PBMC subsets in response to each condition was measured by multi-parameter flow cytometry. Cells were stained with lineage-specific and anti-Ki67 antibodies. The results are expressed as the proportion (%) of Ki67 expressing cells. (A) Schematic diagram of gating strategy to identify each PBMC subpopulation. Ki67 expression in CD3+/CD56- T cells (B), CD19+ B cells (C), CD3-/CD56+ NK cells (D), and CD3+/CD56+ NKT cells (E). Data represents means ± standard errors for 4 subjects. Statistical significance was determined by the Tukey test. *, $P<0.05$; **, $P<0.01$.

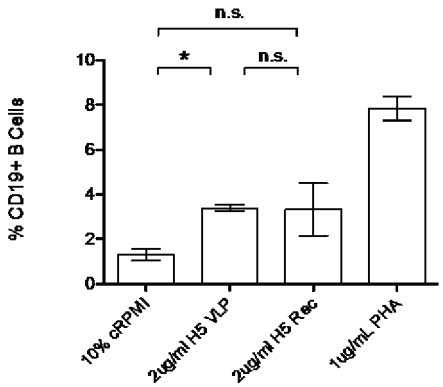
[A]


Total Cell Gate
Live Cell Gate
CD14+ Cell Gate
CD14- Cell Gate
CD3-/CD56+ NK Cells
CD3+/CD56+ NKT Cells
CD19+ B Cells
CD3+/CD56- T Cells

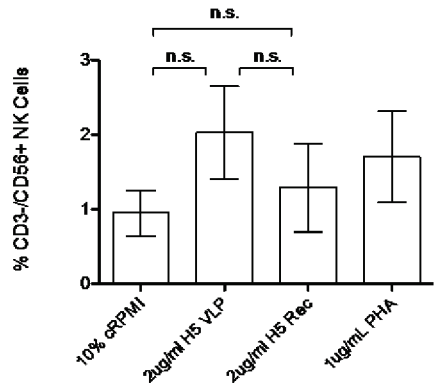
[B]


n.s.
n.s.
n.s.
% CD3+/CD56- T Cells
10% cRPMI
2ug/ml H5 VLP
2ug/ml H5 Rec
1ug/mL PHA

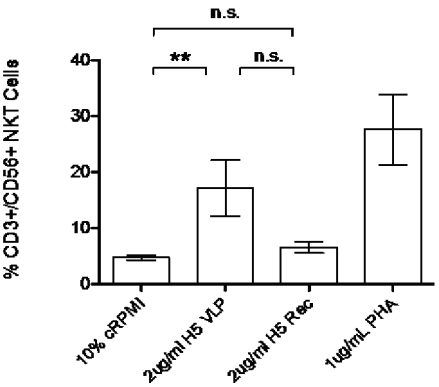
[C]


n.s.
*
n.s.
% CD19+ B Cells
10% cRPMI
2ug/ml H5 VLP
2ug/ml H5 Rec
1ug/mL PHA

[D]

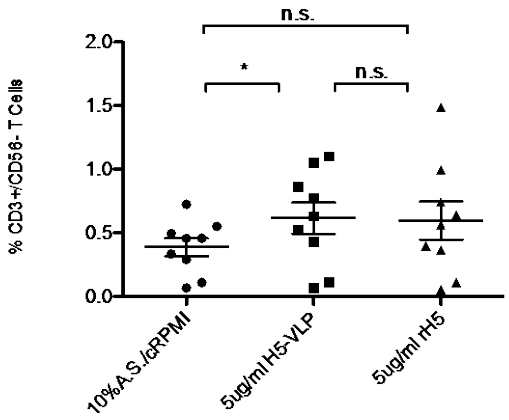

n.s.
n.s.
n.s.
% CD3-/CD56+ NK Cells
10% cRPMI
2ug/ml H5 VLP
2ug/ml H5 Rec
1ug/mL PHA

[E]

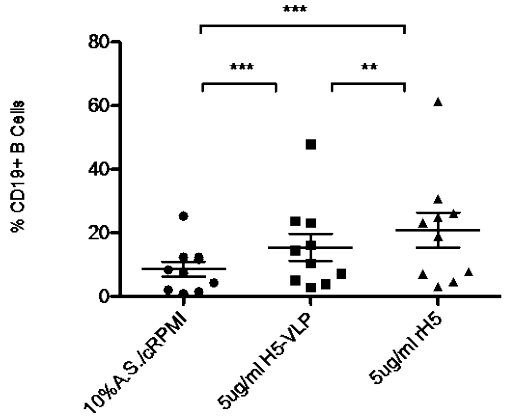

n.s.
**
n.s.
% CD3+/CD56+ NKT Cells
10% cRPMI
2ug/ml H5 VLP
2ug/ml H5 Rec
1ug/mL PHA

2.5.3. Figure 7: Increasing expression of the activation marker CD69 on CD19+ B cells and CD3-/CD56+ NK cells after 6 hours of co-cultured with H5-VLP. Human PBMCs ($5x10^5$ cells/condition) were incubated in the presence of complete RPMI media, H5-VLP or recombinant H5 (both at 5μg/mL) for 6 hours. Cellular activation of each PBMC subset was measured by multi-parameter flow cytometry. PBMCs were stained with lineage-specific antibodies and anti-CD69. The results are expressed as the proportion (%) of CD69 expressing cells. The gating strategy used for each PBMC subset was identical to that shown in Figure 6. The results show CD69 expression in CD3+/CD56- T cells (A), CD19+ B cells (B), CD3-/CD56+ NK cells (C), and CD14+ cells (D). Data represent mean ± standard errors for 7 to 10 subjects. Statistical significance was determined by the Mann-Whitney test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

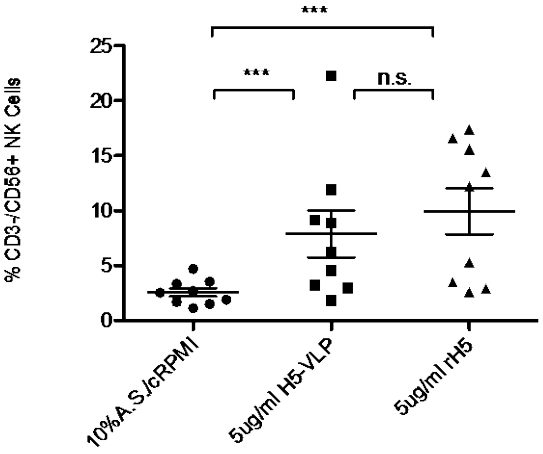
[A]


n.s.
*
n.s.
% CD3+/CD56- T Cells
2.0
1.5
1.0
0.5
0.0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

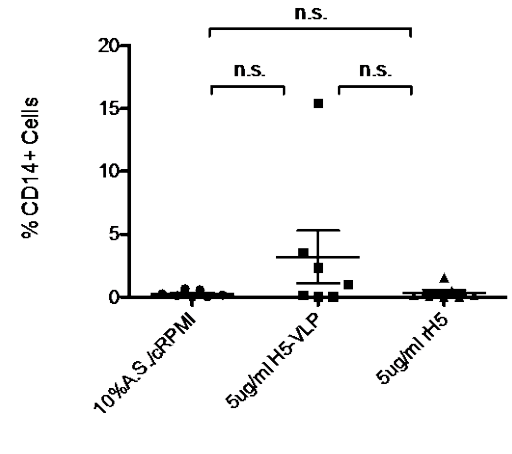
[B]


***
***
**
% CD19+ B Cells
80
60
40
20
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

[C]

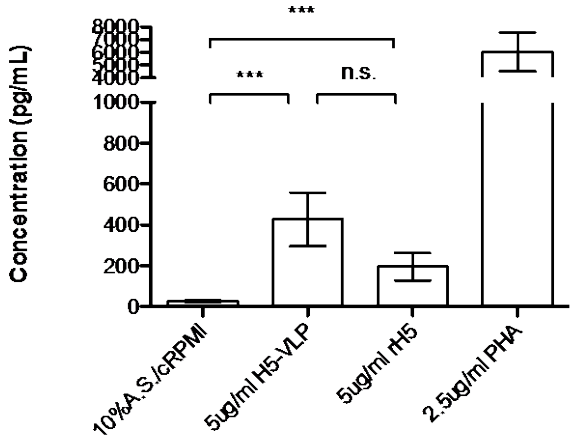

***
***
n.s.
% CD3-/CD56+ NK Cells
25
20
15
10
5
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

[D]

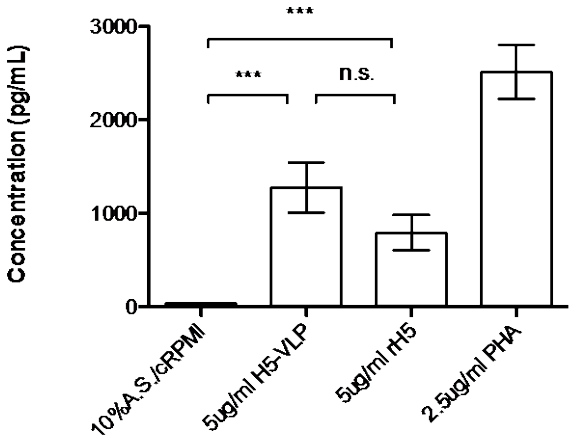

n.s.
n.s.
n.s.
% CD14+ Cells
20
15
10
5
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

2.5.4. Figure 8: Human PBMCs co-cultured with H5-VLP secrete predominantly pro-inflammatory cytokines. Human PBMCs ($2x10^5$ cells/well) were co-cultured with complete RPMI media (10% cRPMI), H5-VLP (5μg/mL), recombinant H5 (rH5; 5μg/mL), or PHA (2.5μg/mL) for 6 hours. Supernatants were collected and cytokine concentrations were measured by multiplex enzyme-linked immunosorbant assay (ELISA): (A) IL-1β, (B) IL-6, (C) IL-8, (D) TNF-α. Cytokine secretion patterns by PBMCs co-cultured with H5-VLP for 6, 24, and 48 hours are shown in Figure: (E) IL-1β, and (F) TNF-α. Data represent mean ± standard error for 10 subjects. Statistical significance was determined by the Mann-Whitney test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

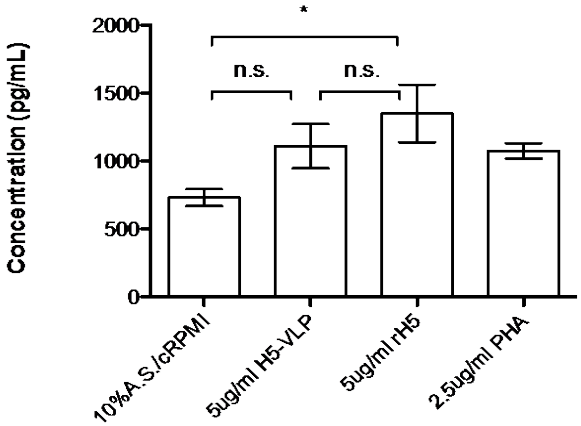
[A]


***
***
n.s.
Concentration (pg/mL)
8000
7000
6000
5000
4000
1000
800
600
400
200
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5
2.5ug/ml PHA

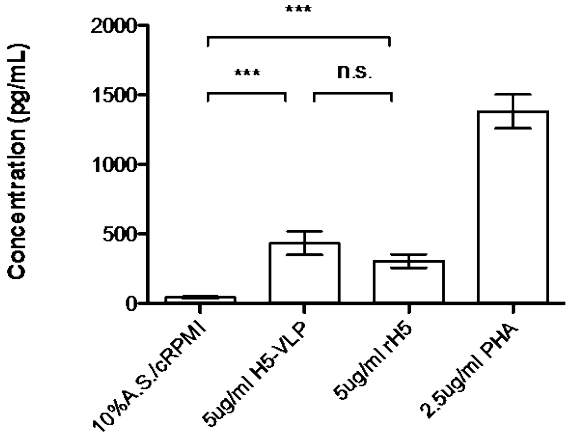
[B]


***
***
n.s.
Concentration (pg/mL)
3000
2000
1000
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5
2.5ug/ml PHA

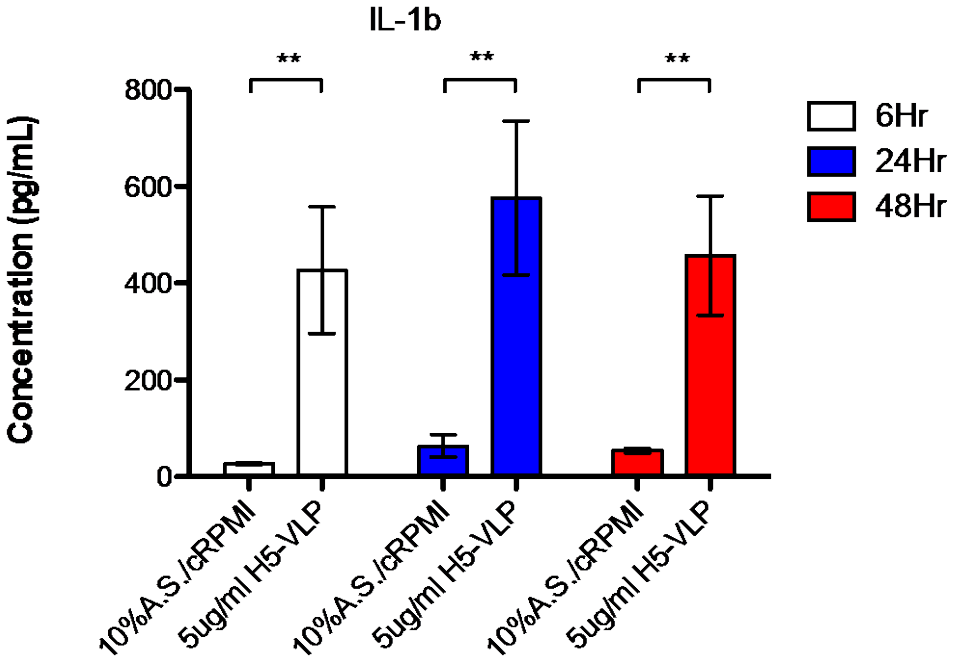
[C]


*
n.s.
n.s.
Concentration (pg/mL)
2000
1500
1000
500
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5
2.5ug/ml PHA

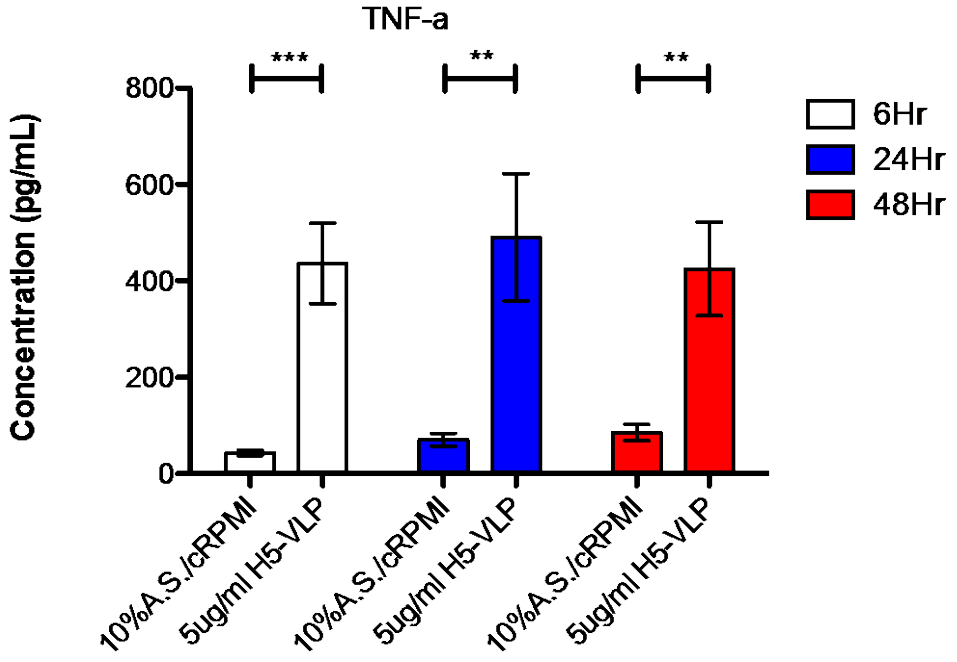
[D]


***
***
n.s.
Concentration (pg/mL)
2000
1500
1000
500
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5
2.5ug/ml PHA

[E]

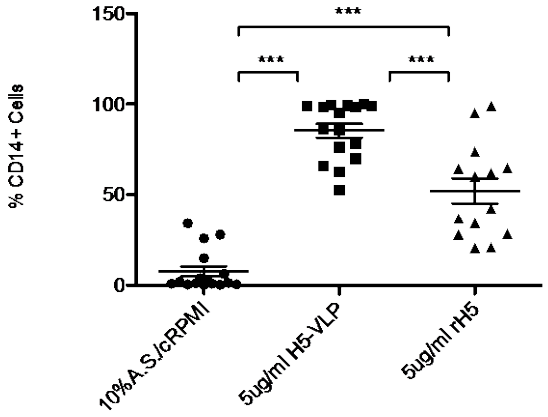

IL-1b
**
**
**
800
600
400
200
0
Concentration (pg/mL)
6Hr
24Hr
48Hr
10%A.S./cRPMI
5ug/ml H5-VLP
10%A.S./cRPMI
5ug/ml H5-VLP
10%A.S./cRPMI
5ug/ml H5-VLP

[F]

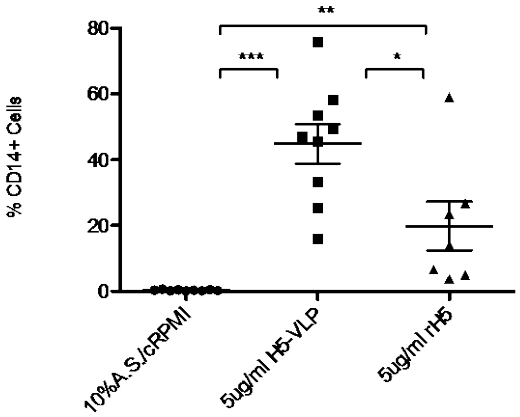

TNF-a
***
**
**
800
600
400
200
0
Concentration (pg/mL)
6Hr
24Hr
48Hr
10%A.S./cRPMI
5ug/ml H5-VLP
10%A.S./cRPMI
5ug/ml H5-VLP
10%A.S./cRPMI
5ug/ml H5-VLP

2.5.5. Figure 9: Increased expression of pro-inflammatory cytokines was observed in human CD14+ cells co-cultured with H5-VLP. Human PBMCs ($5x10^5$ cells/condition) were co-cultured with complete RPMI media (10% cRPMI), H5-VLP, or recombinant H5 (both at 5μg/mL) for 6 hours. The proportion (%) of CD14+ cells expressing the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α were measured by flow cytometry. The results are expressed as percent cytokine-expressing cells: (A) IL-1β, (B) IL-6, (C) IL-8, and (D) TNF-α. (E) A representative flow cytometric scatter plot of IL-1β, IL-6, IL-8, and TNF-α expressed by CD14+ cells 6 hours after stimulation with complete RPMI media ([i] and [iv]), H5-VLP ([ii] and [v]), and recombinant H5 ([iii] and [vi]). Data represent mean ± standard errors for 7 to 16 subjects. Statistical significance was determined by the Mann-Whitney test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

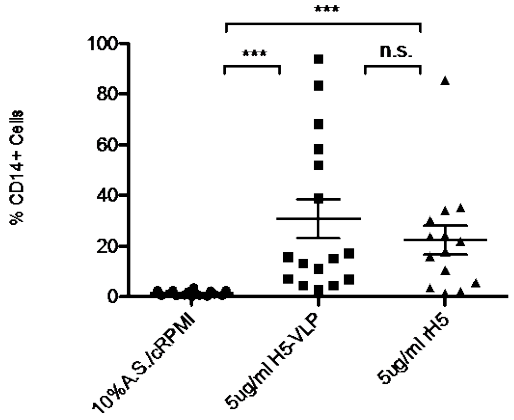
[A]


% CD14+ Cells
150
100
50
0
***
***
***
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

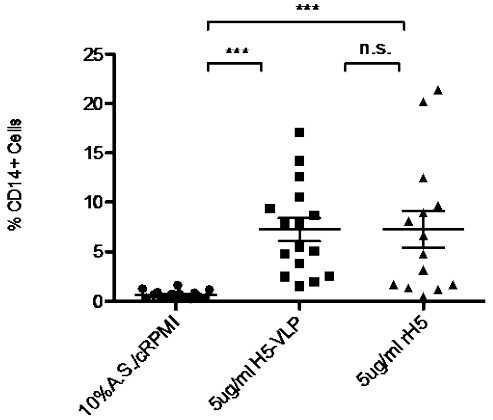
[B]


% CD14+ Cells
80
60
40
20
0
**
***
*
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

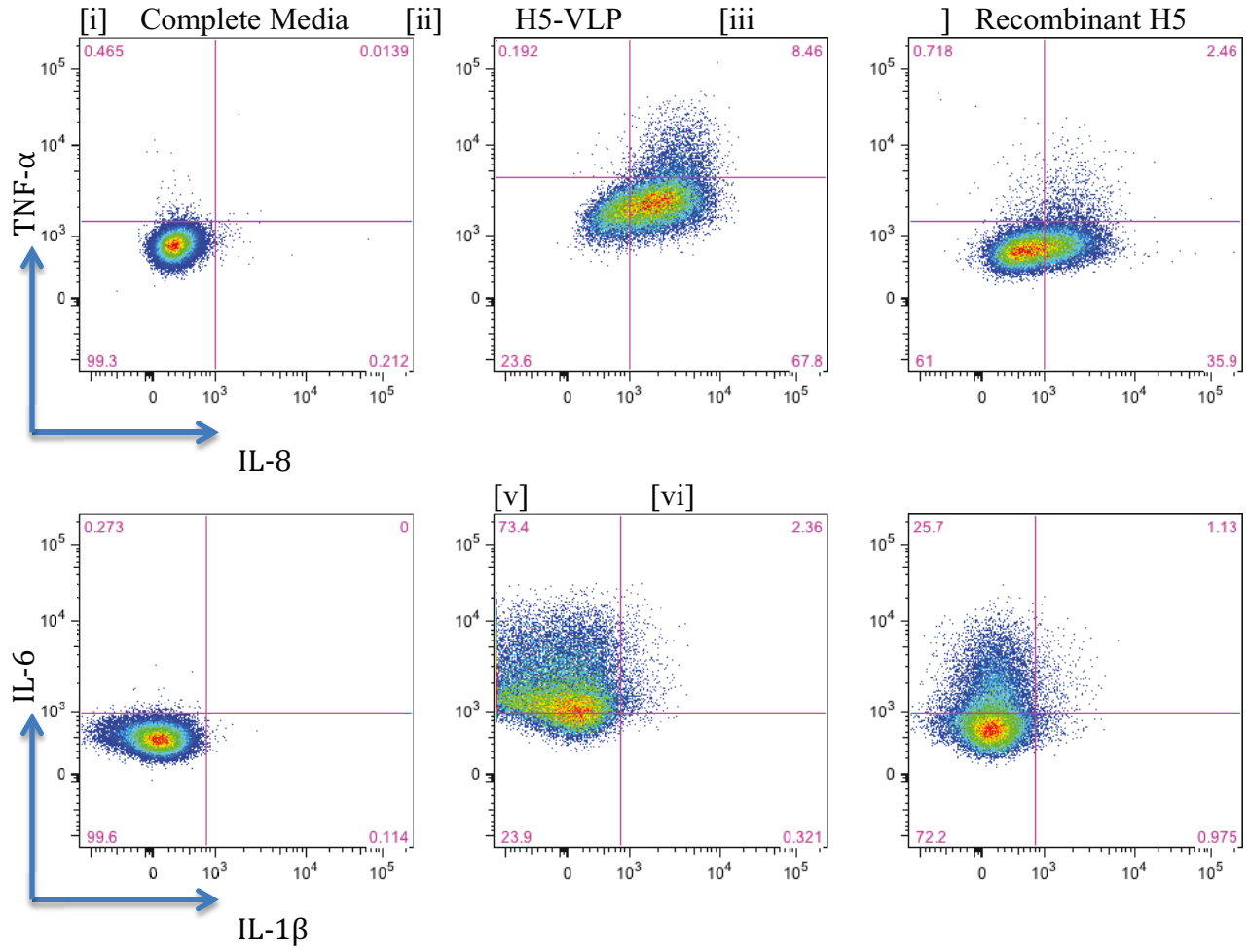
[C]


% CD14+ Cells
100
80
60
40
20
0
***
***
n.s.
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

[D]

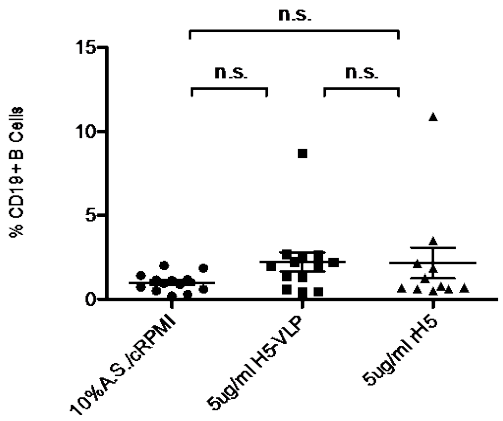

% CD14+ Cells
25
20
15
10
5
0
***
***
n.s.
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

[E]

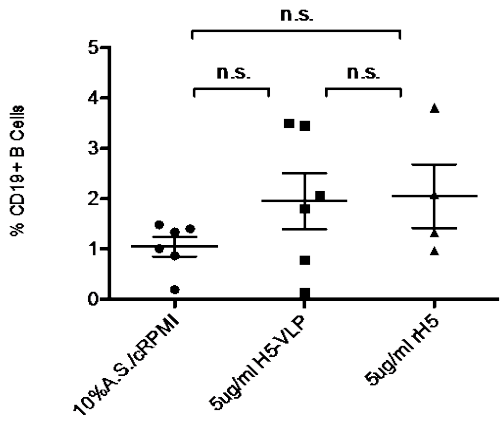

[i] Complete Media
[ii] H5-VLP
[iii] Recombinant H5
[iv]
[v]
[vi]
TNF-α
IL-8
IL-6
IL-1β
0.465
0.0139
99.3
0.212
0.192
8.46
23.6
67.8
0.718
2.46
61
35.9
0.273
0
99.6
0.114
73.4
2.36
23.9
0.321
25.7
1.13
72.2
0.975

2.5.6. Figure 10: No significant increase of pro-inflammatory cytokine expression in human CD19+ B cells co-cultured with H5-VLP. Human PBMCs ($5x10^5$ cells/condition) were co-cultured with complete RPMI media (10% cRPMI), H5-VLP, or recombinant H5 (both at 5μg/mL) for 6 hours. The proportion (%) of CD19+ B cells expressing IL-1β, IL-6, IL-8 and TNF-α were measured by flow cytometry. The results are expressed as a percent of cytokine-expressing cells: (A) IL-1β, (B) IL-6, (C) IL-8, and (D) TNF-α. (E) A representative flow cytometric scatter plot of IL-1β, IL-6, IL-8, and TNF-α expression by CD19+ B cells at 6 hours after stimulation by complete RPMI media ([i] and [iv]), H5-VLP ([ii] and [v]), and recombinant H5 ([iii] and [vi]). Data represent mean ± standard errors for 6 to 13 subjects. Statistical significance was determined by the Mann-Whitney test. *, $P<0.05$; ***, $P<0.01$; ***, $P<0.001$.

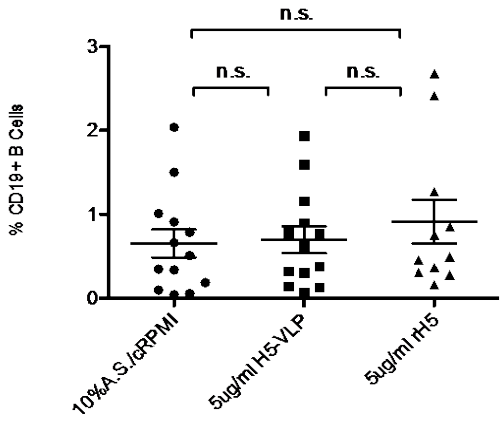
[A]


n.s.
n.s.
n.s.
% CD19+ B Cells
15
10
5
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

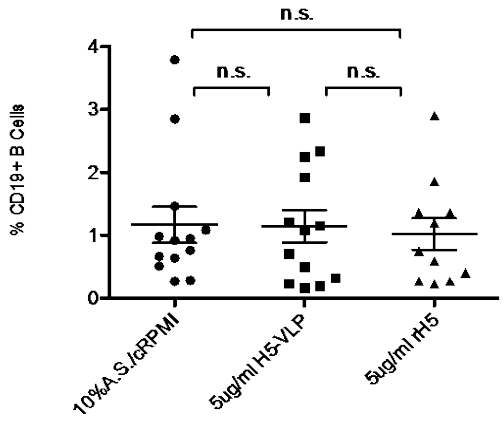
[B]


n.s.
n.s.
n.s.
% CD19+ B Cells
5
4
3
2
1
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

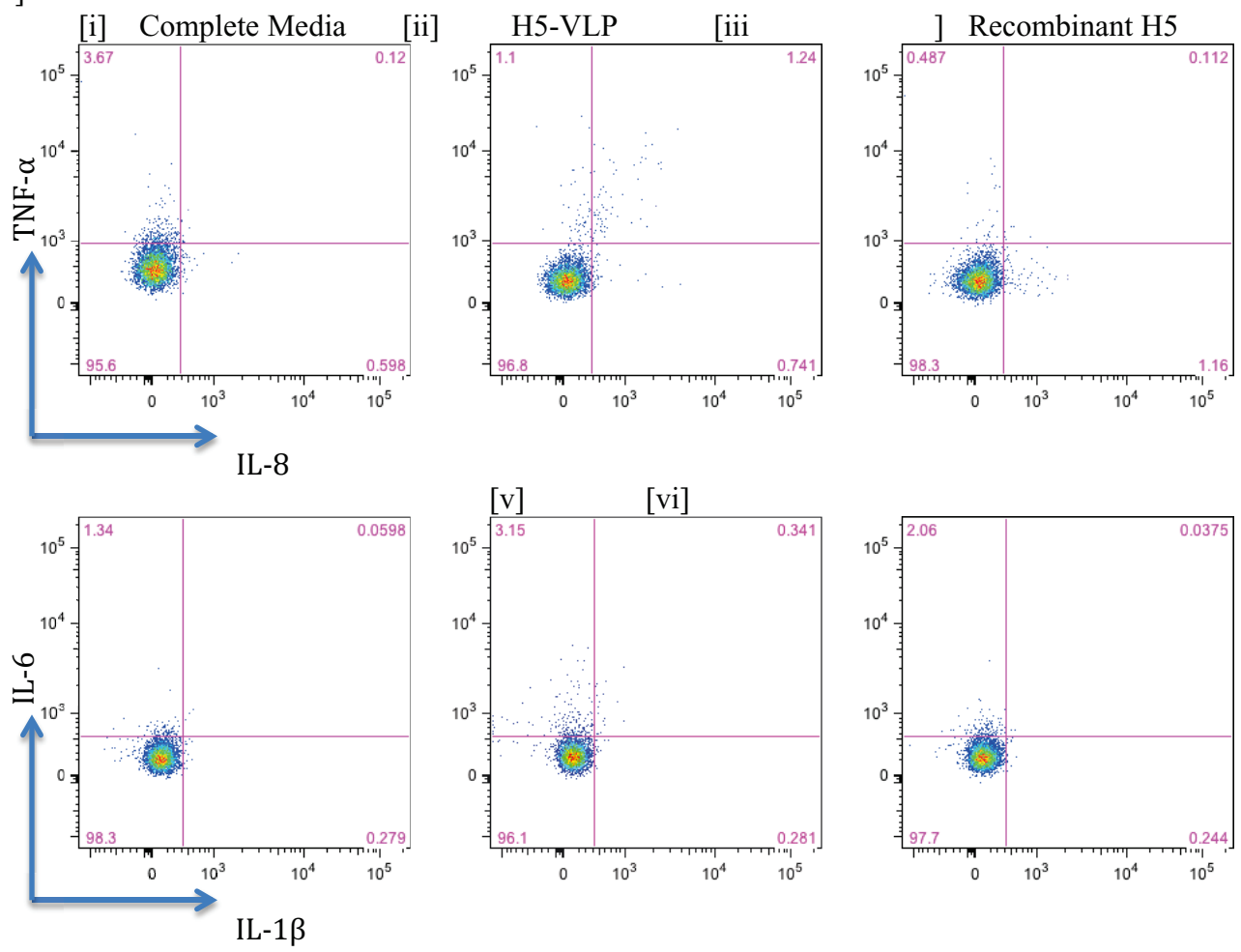
[C]


n.s.
n.s.
n.s.
% CD19+ B Cells
3
2
1
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

[D]

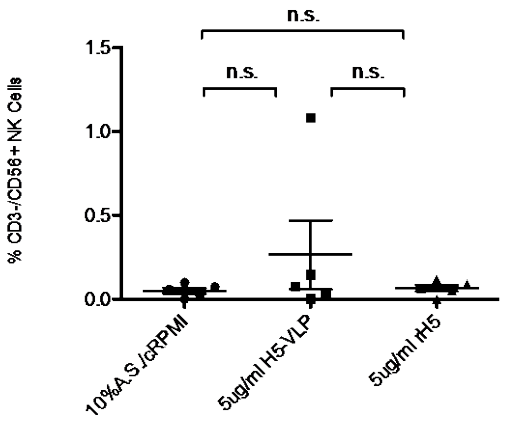

n.s.
n.s.
n.s.
% CD19+ B Cells
4
3
2
1
0
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

[E]

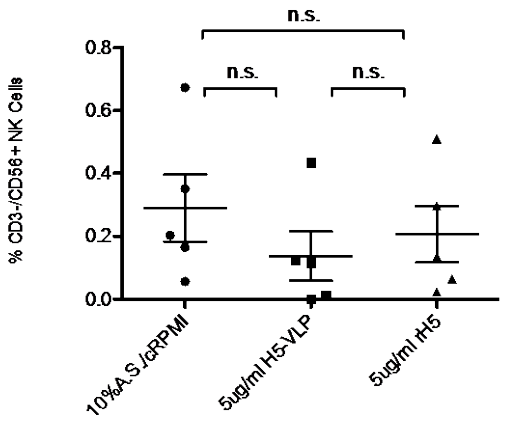

[i] Complete Media
[ii] H5-VLP
[iii] Recombinant H5
TNF-α
IL-8
3.67
0.12
95.6
0.598
1.1
1.24
96.8
0.741
0.487
0.112
98.3
1.16
[iv]
[v]
[vi]
IL-6
IL-1β
1.34
0.0598
98.3
0.279
3.15
0.341
96.1
0.281
2.06
0.0375
97.7
0.244

2.5.7. Figure 11: No significant increase of pro-inflammatory cytokine expression in human CD3-/CD56+ NK cells co-cultured with H5-VLP. Human PBMCs ($5x10^5$ cells/condition) were co-cultured with complete RPMI media (10% cRPMI), H5-VLP, or recombinant H5 (both at 5μg/mL) for 6 hours. The proportion (%) of CD3-/CD56+ NK cells expressing IL-1β, IL-6, IL-8 and TNF-α were measured by flow cytometry. The results are expressed as the percent of cytokine-expressing cells: (A) IL-1β, (B) IL-6, (C) IL-8, and (D) TNF-α. (E) A representative flow cytometric scatter plot of IL-1β, IL-6, IL-8, and TNF-α expressing by CD3-/CD56+ NK cells at 6 hours after stimulation with complete RPMI media ([i] and [iv]), H5-VLP ([ii] and [v]), and recombinant H5 ([iii] and [vi]). Data represent mean ± standard errors for 4 to 5 subjects. Statistical significance was determined by the Mann-Whitney test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

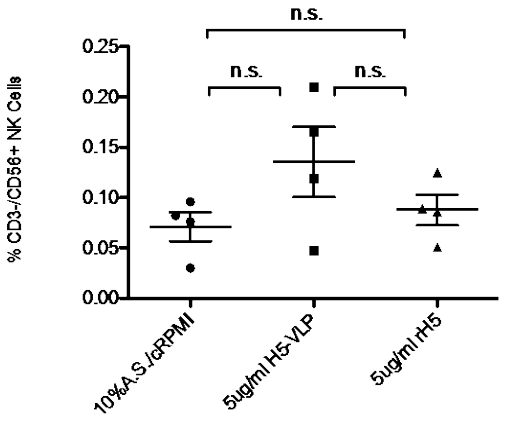
[A]


n.s.
n.s.
n.s.
1.5
1.0
0.5
0.0
% CD3-/CD56+ NK Cells
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

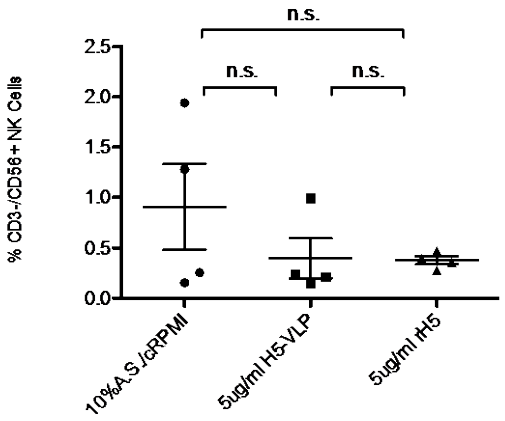
[B]


n.s.
n.s.
n.s.
0.8
0.6
0.4
0.2
0.0
% CD3-/CD56+ NK Cells
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

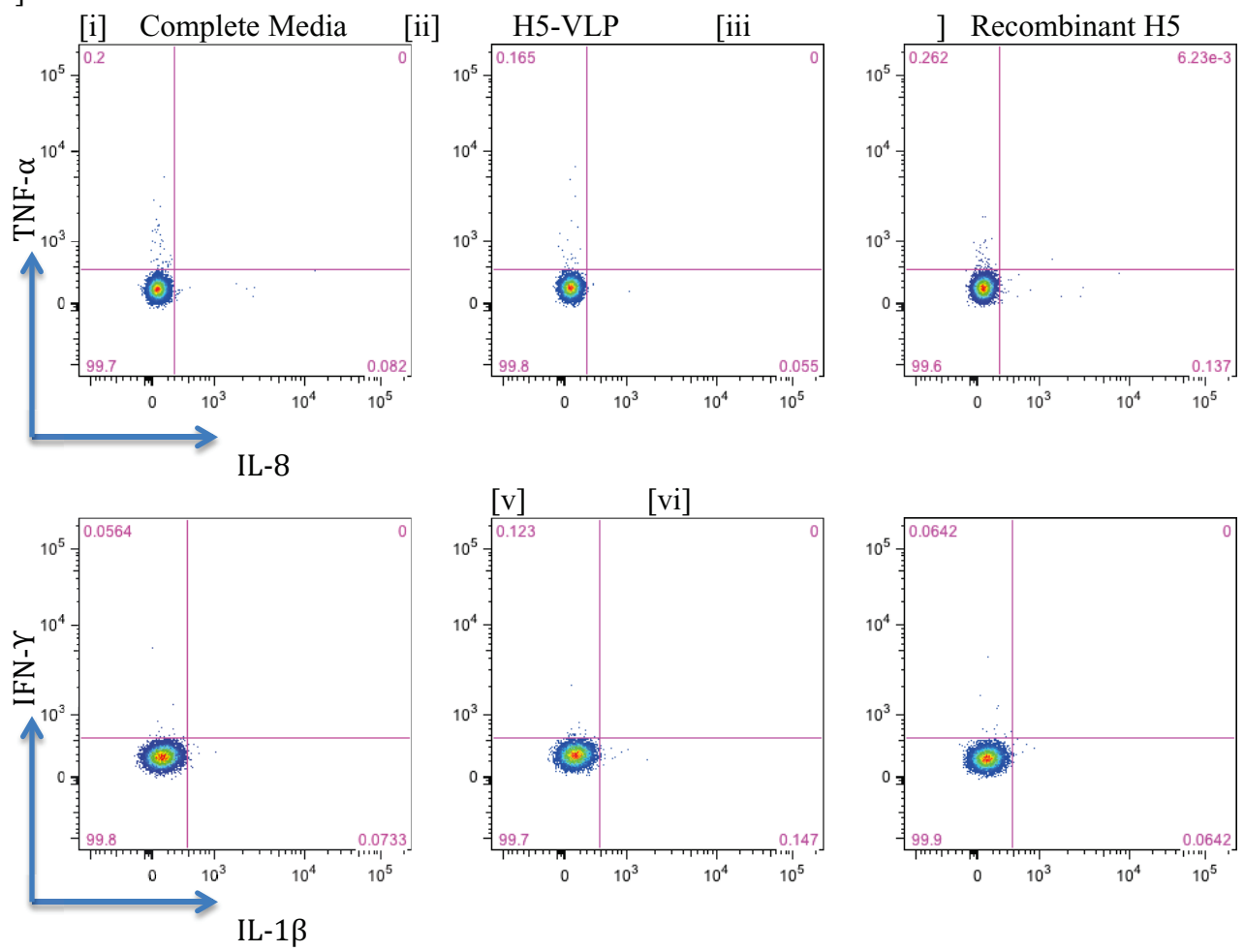
[C]


n.s.
n.s.
n.s.
0.25
0.20
0.15
0.10
0.05
0.00
% CD3-/CD56+ NK Cells
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

[D]

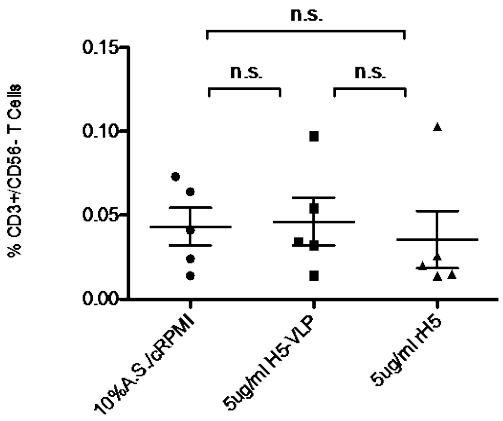

n.s.
n.s.
n.s.
2.5
2.0
1.5
1.0
0.5
0.0
% CD3-/CD56+ NK Cells
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

[E]

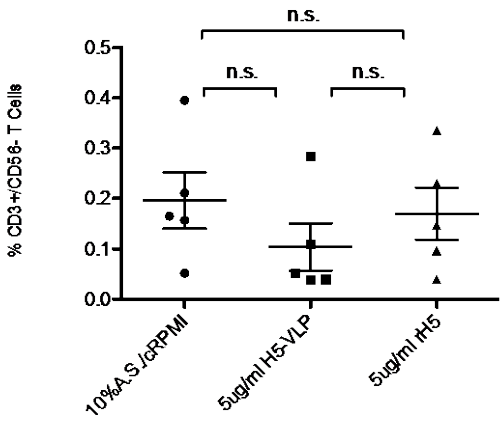

[i] Complete Media
[ii] H5-VLP
[iii] Recombinant H5
TNF-α
IL-8
0.2
0
99.7
0.082
0.165
0
99.8
0.055
0.262
6.23e-3
99.6
0.137
[iv]
[v]
[vi]
IFN-Y
IL-1β
0.0564
0
99.8
0.0733
0.123
0
99.7
0.147
0.0642
0
99.9
0.0642

2.5.8. Figure 12: No significant increase of pro-inflammatory cytokine expression in human CD3+/CD56+ T cells co-cultured with H5-VLP. Human PBMCs ($5x10^5$ cells/condition) were co-cultured with complete RPMI media (10% cRPMI), H5-VLP, or recombinant H5 (both at 5μg/mL) for 6 hours. The proportion (%) of CD3+/CD56- T cells expressing IL-1β, IL-6, IL-8 and TNF-α were measured by flow cytometry. The results are expressed as the percent of cytokine-expressing cells: (A) IL-1β, (B) IL-6, (C) IL-8, and (D) TNF-α. (E) A representative flow cytometric scatter plot of IL-1β, IL-6, IL-8, and TNF-α expressed by CD19+ B cells at 6 hours after stimulation with complete RPMI media ([i] and [iv]), H5-VLP ([ii] and [v]), and recombinant H5 ([iii] and [vi]). Data represent mean ± standard errors for 4 to 5 subjects. Statistical significance was determined by the Mann-Whitney test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

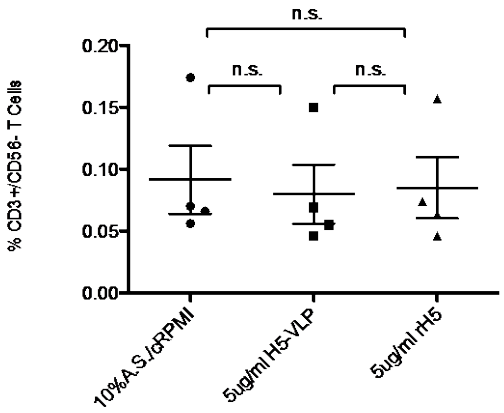
[A]


n.s.
n.s.
n.s.
0.15
0.10
0.05
0.00
% CD3+/CD56- T Cells
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

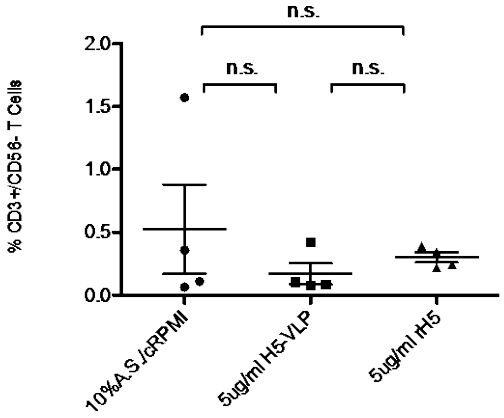
[B]


n.s.
n.s.
n.s.
0.5
0.4
0.3
0.2
0.1
0.0
% CD3+/CD56- T Cells
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

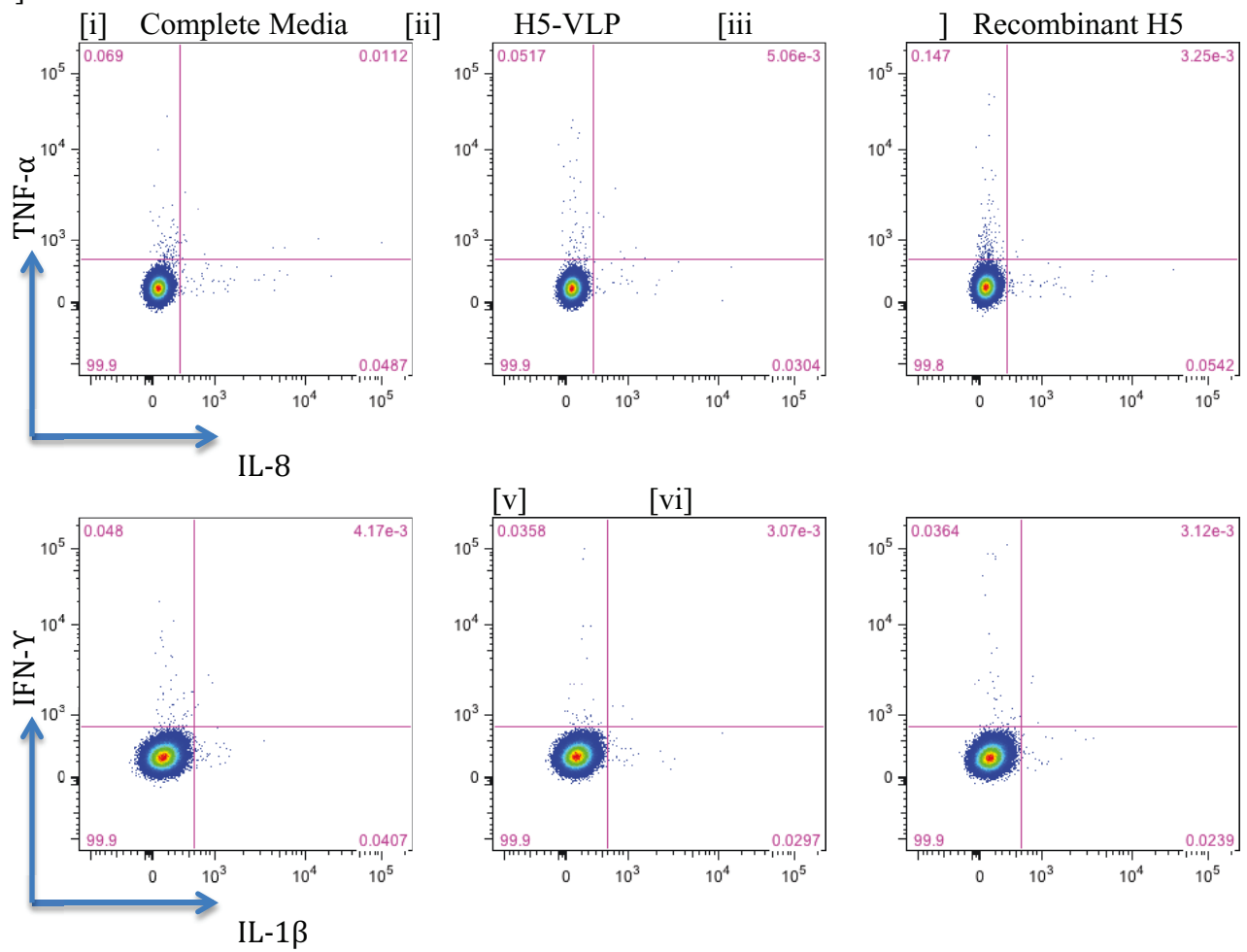
[C]


n.s.
n.s.
n.s.
0.20
0.15
0.10
0.05
0.00
% CD3+/CD56- T Cells
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

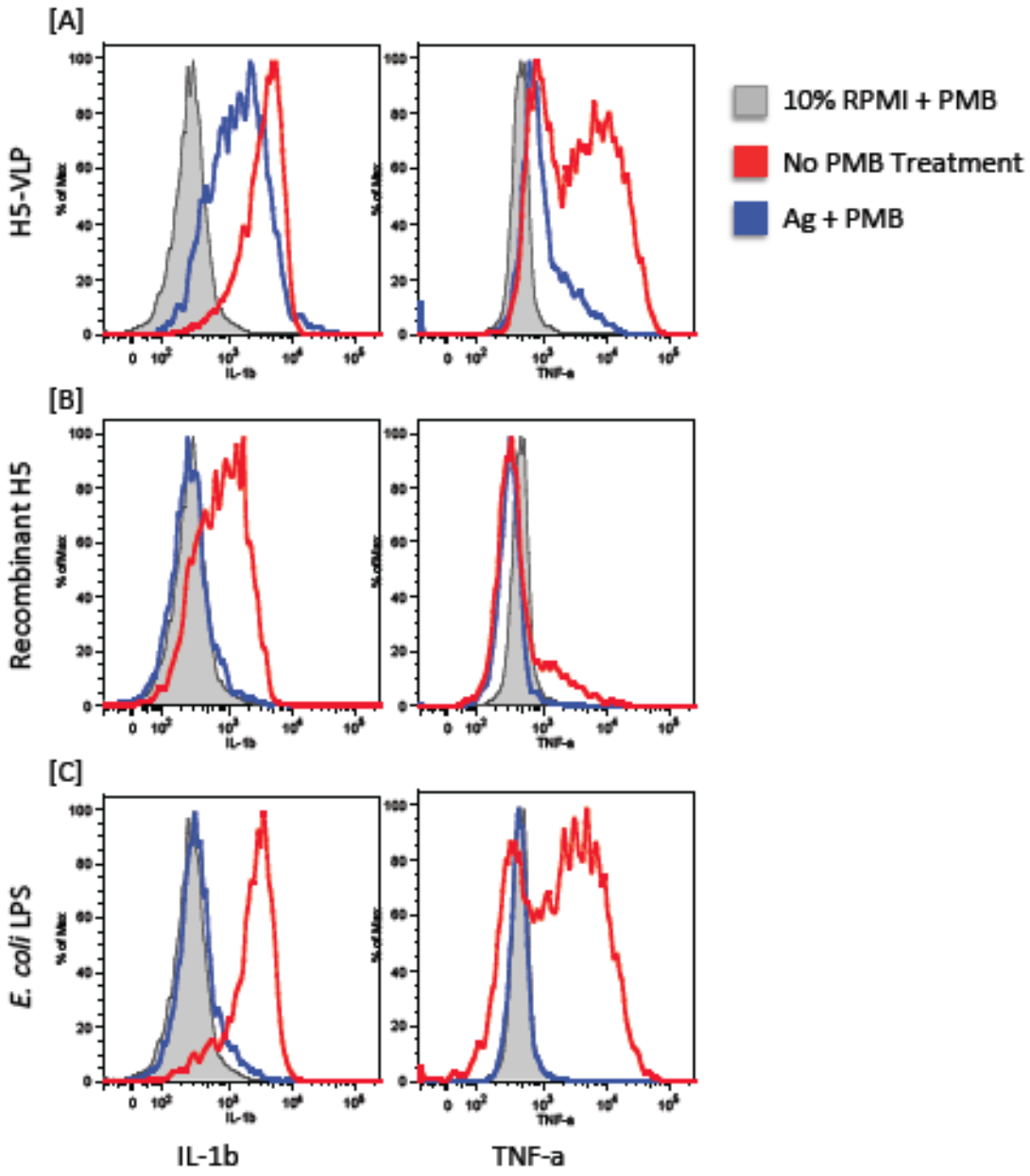
[D]


n.s.
n.s.
n.s.
2.0
1.5
1.0
0.5
0.0
% CD3+/CD56- T Cells
10%A.S./cRPMI
5ug/ml H5-VLP
5ug/ml rH5

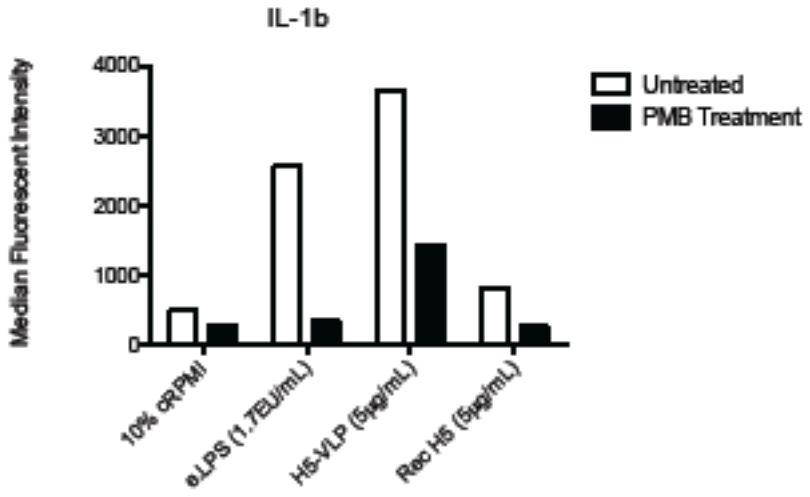
[E]


[i] Complete Media
[ii] H5-VLP
[iii] Recombinant H5
TNF-α
IL-8
0.069
0.0112
99.9
0.0487
0.0517
5.06e-3
99.9
0.0304
0.147
3.25e-3
99.8
0.0542
[iv]
[v]
[vi]
IFN-γ
IL-1β
0.048
4.17e-3
99.9
0.0407
0.0358
3.07e-3
99.9
0.0297
0.0364
3.12e-3
99.9
0.0239

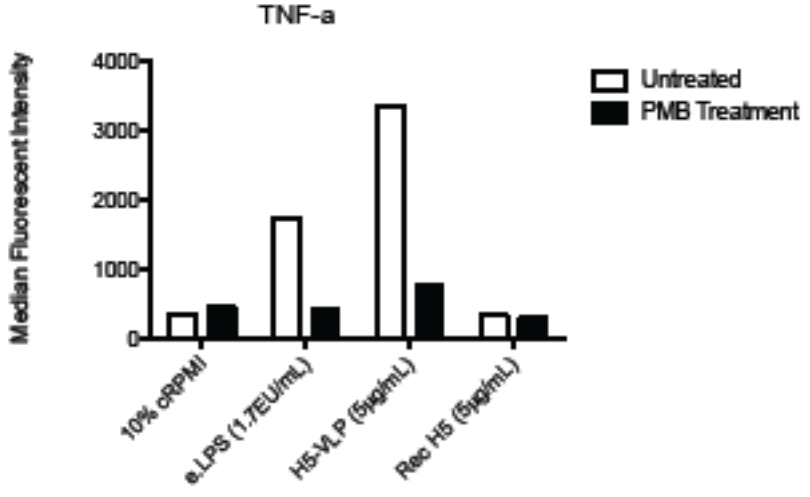
2.5.9. Figure 13: Plant-derived H5-VLP can still induce IL-1β and TNF-α production even after removal of *A. tumefaciens*-derived LPS. Human PBMCs ($5x10^5$ cells/condition) were co-cultured with 10% complete RPMI media, H5-VLP (5μg/mL), *E. coli*-derived LPS (1.7EU/mL), and recombinant H5 (5μg/mL), pre-treated or not with polymyxin B (PMB; 160μg/mL) for 3 hours. The expression of IL-1β, IL-6, IL-8 and TNF-α in CD14+ cells was measured by flow cytometry. The results (A-C) are presented in histogram from: (A) PBMCs stimulated with H5-VLP; (B) PBMCs stimulated with recombinant H5; and (C) PBMC stimulated with *E. coli* LPS. Grey: Media+PMB negative control; Red: Antigen **without** PMB treatment; Blue: Antigen **with** PMB treatment. The median fluorescent intensities (MFI) of IL-1β and TNF-α are depicted as bar graphs: (D) MFI of IL-1β in CD14+ cells under various conditions; and (E) MFI of TNF-α in CD14+ cells under various conditions. This experiment was repeated twice with PBMC from a single subject.


[A]
[B]
[C]
H5-VLP
Recombinant H5
E. coli LPS
IL-1b
TNF-a
10% RPMI + PMB
No PMB Treatment
Ag + PMB

[D]


IL-1b
Median Fluorescent Intensity
4000
3000
2000
1000
0
Untreated
PMB Treatment
10% cRPMI
eLPS (1.7EU/mL)
H5-VLP (5µg/mL)
Rec H5 (5µg/mL)

[E]


TNF-a
Median Fluorescent Intensity
4000
3000
2000
1000
0
Untreated
PMB Treatment
10% cRPMI
eLPS (1.7EU/mL)
H5-VLP (5µg/mL)
Rec H5 (5µg/mL)

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