Internalization and endosomal trafficking of hemagglutinin-bearing virus-like particles favors antigen presentation in human macrophages

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CONTRIBUTION ON AUTHORS

I planned and conducted all the experiments and data analyses included in this thesis, with the exceptions listed below.

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ABBREVIATIONS

ANOVA _____ Analysis of variance

BC _____ Before Christ

BCR _____ B-Cell Receptor

CCD _____ Charge-Coupled Device

CCEE _____ Clathrin-Coated Early Endosome

CCV _____ Clathrin-Coated Vesicle

CDC _____ Centers for Disease Control and prevention

Cdc42 _____ Cell division control protein 42

CIE _____ Clathrin-Independent Endocytosis

CME _____ Clathrin-Mediated Endocytosis

CO2 _____ Carbon dioxide

cRNA _____ Complementary ribonucleic acid

DAPI _____ 4′,6-diamidino-2-phenylindole

DC _____ Dendritic Cell

DNA _____ Deoxyribonucleic acid

EE _____ Early Endosome

EEA1 _____ Early Endosome Antigen 1

ER _____ Endoplasmic reticulum

EV _____ Endocytic vesicle

FBS _____ Fetal Bovine Serum

H(number)_____ Hemagglutinin (number)

H(number)N(number)_____ Hemagglutinin (number) Neuraminidase (number)

HA_____ Hemagglutinin

HEPES _____ 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HL _____ Heterolysosome

IAV _____ Influenza A Virus

IBV _____ Influenza B Virus

ICV _____ Influenza C Virus

IDV _____ Influenza D Virus

IgG _____ Immunoglobulin G

IIV _____ Inactivated Influenza Virus

IU _____ International Unit

LAIV _____ Live Attenuated Influenza Virus

LAMP1 _____ Lysosomal-Associated Membrane Protein 1

LPS _____ Lipopolysaccharide

M1 _____ Matrix protein 1

M2 _____ Matrix protein 2

Mab _____ Monoclonal antibody

MDM_____Monocyte-Derived Macrophage

MHC _____ Major Histocompatibility Complex

mRNA_____ Messenger ribonucleic acid

MUHC_____ McGill University Health Centre

MVB _____Multi-Vesicular Body

N(number)_____Neuraminidase(number)

NA_____ Neuraminidase

NEP _____Nuclear Export Protein

NP _____Nucleoprotein

NS _____ Nonstructural protein

NS1 _____Nonstructural protein 1

NS2 _____Nonstructural protein 2

PA _____ Polymerase Acidic protein

PABP1_____ Poly(A)-Binding Protein 1

PB1 _____ Polymerase Basic protein 1

PB2 _____ Polymerase Basic protein 2

PBMC _____ Peripheral Blood Mononuclear Cell

PBS _____ Phosphate Buffered Saline

pH _____ Potential Hydrogen

pMHC _____ Peptide-loaded Major Histocompatibility Complex

QIV _____ Quadrivalent Influenza Vaccine

Rab _____ Ras-associated binding protein

Rac1 _____ Ras-related C3 botulinum toxin substrate 1

RIV _____ Recombinant Influenza Virus

RNA _____ Ribonucleic Acid

RNP _____ Ribonucleoprotein

ROI _____ Region Of Interest

RPMI _____ Roswell Park Memorial Institute

TCR _____ T-Cell Receptor

TEM _____ Transmission Electron Microscopy

TIV _____ Trivalent Influenza Vaccine

UnCV _____ Uncoated Vesicles

VE _____ Vaccine efficacy

VLP _____ Virus-Like Particle

vRNA _____ Viral Ribonucleic Acid

vRNP _____ Viral Ribonucleoprotein

WHO _____ World Health Organization

1. ABSTRACT AND RÉSUMÉ

1.1. Abstract

Influenza is a respiratory tract infection caused by influenza viruses. The most effective way to prevent this disease is through vaccination. Despite their availability, licensed influenza vaccines still have several limitations that cause inconsistent vaccine efficacy every year, as the influenza viruses mutate and generate new viral strains, and these vaccines provide poor protection to more susceptible population groups such as the elderly. Thus, influenza remains a social, economic, and health burden every year, causing substantial numbers of severe illness cases and deaths, worldwide. To overcome these limitations, new vaccination approaches are being currently researched.

A promising candidate for influenza vaccination are virus-like particles (VLPs) bearing hemagglutinin developed by Medicago Inc. These particles are not replicative as they lack genetic material, and they have been shown to recapitulate viral interactions with the immune system. More importantly, these particles have been shown to elicit strong and cross-reactive humoral and cell-mediated immune response in clinical studies. However, the unique and potent immune responses generated by Medicago's VLPs are still not completely understood.

In this study, we used VLPs bearing hemagglutinin derived from an H1N1 strain and human monocyte-derived macrophages to study the VLPs interactions with antigen presenting cells. We demonstrate that these particles are first internalized via clathrin-mediated endocytosis, caveolin-dependent endocytosis, and micropinocytosis, and then they are delivered to early endosomes. From this compartment, hemagglutinin can be directed to both lysosomal and recycling compartments for protein degradation. Both the diverse mechanisms of internalization of these VLPs and their different possible intracellular pathways indicate that the hemagglutinin carried by

these particles may end up being degraded in compartments associated with MHC I and MHC II antigen presentation.

These results suggest that the diverse way in which the VLPs are handled by antigen presenting cells may results in both MHC I and MHC II presentation that elicit the response of CD8+ and CD4+ T cells, respectively, which are in charge of coordinating the immune response and generating immune memory. Our results help explain how Medicago's VLPs are a powerful candidate for influenza vaccination as they elicit a varied immune responses, in contrast with other available vaccines.

1.2. Résumé

La grippe ou l'influenza est une infection respiratoire causée par les virus grippaux. La façon la plus efficace de prévenir cette maladie est par la vaccination. Malgré leur accessibilité, les vaccins contre l'influenza ont encore quelques limites causant une efficacité variable à chaque année, puisque les virus de la grippe mutent et de nouvelles souches de la maladie sont générées apportant ainsi une faible protection aux groupes les plus vulnérables de la société tels que les personnes âgées. Ainsi, à chaque année, l'influenza demeure un problème social, économique et sanitaire causant un nombre élevé de malades sévères et de morts partout sur la planète. Pour enrayer la maladie, de nouvelles approches sont sous investigation.

Un candidat prometteur dans le secteur vaccins grippaux sont les particules pseudovirales (PPV) présentant l'hémagglutinine fabriquées par Medicago Inc. Les particules ne sont pas réplicatives faute de matériel génétique mais elles récapitulent les interactions virales avec le système immunitaire. Qui plus est, ces particules stimulent de fortes réponses humorales et à médiation cellulaire immunitaire croisées lors d'essais cliniques. Toutefois, la puissante et unique réponse immunitaire induite par les PPV de Medicago ne sont pas entièrement compris.

Dans cette étude, nous utilisons des PPV présentant l'hémagglutinine dérivée d'une souche H1N1 et des macrophages dérivés de monocytes humains afin d'analyser les interactions des PPV avec des cellules présentatrices d'antigènes. Nous avons démontré que ces particules sont d'abord intériorisées par une endocytose médiée par la clathrine, une endocytose dépendante de la cavéoline et la macropinocytose, puis transportées aux endosomes précoces. De ce compartiment, l'hémagglutinine véhiculée par ces particules peut se diriger vers les compartiments lysosomaux et de recyclage pour la dégradation des protéines. À la fois les différents mécanismes d'internalisation de ces PPV ainsi que leurs possibles voies intracellulaires différentes indiquent

que l'hémagglutinine véhiculée par ces particules peut éventuellement se dégrader dans des compartiments associés à la présentation d'antigène via les CMH I et CMH II.

Ces résultats suggèrent que les diverses manières dont les PPV sont traitées par les cellules présentatrices d'antigènes peuvent avoir pour résultat, à la fois, les présentations de CMH I et de CMH II, qui provoquent respectivement la réponse des cellules T CD8 + et CD4 +, qui coordonnent la réponse immunitaire et générer une mémoire immunitaire. Nos résultats aident à expliquer en quoi les PPV de Medicago sont un candidat puissant pour la vaccination antigrippale, car elles induisent des réponses immunitaires variées, contrairement aux autres vaccins disponibles.

2. LITERATURE REVIEW AND INTRODUCTION

2.1. Influenza

Influenza (commonly known as "the flu") is a respiratory tract infection caused by influenza viruses. Although the symptoms may vary, influenza infection generally causes fever, weakness, muscular pain, cough, and nasal congestion (Monto *et al.*, 2000). Influenza infections present a health and socio-economic problem at present (WHO, 2018).

2.2. History of influenza

Even though the earliest historical description of influenza-like epidemics is too vague to be reliably used as a diagnostic, some authors consider that the first records of influenza are from the 5th century BC (Arenilla, 1985). During the Middle Ages, there were more recorded cases of influenza outbreaks in Europe (Vaughan, 1921). Between the 18th and 20th century, other epidemic and probably pandemic outbreaks occurred (Beveridge, 1991).

It was not until 1918 when the first well-documented pandemic event of influenza struck: the so-called 'Spanish' flu. It caused an estimate of 50 million deaths worldwide (Johnson and Mueller, 2002). J.S. Koen coined the term "influenza" (from Latin, *influentia* – influence) this same year, after observing what he considered to be the same disease (influenza) inter-infecting humans and pigs (Broxmeyer, 2006).

Initially, Pfeiffer had described influenza as a bacterial infection (Taubenberger 2007). Only after the Spanish flu pandemic, Shope demonstrated the disease was in fact caused by a virus (Lewis and Shope, 1931; Shope, 1931-1; Shope, 1931-2).

After the Spanish flu and until the early 21st century, other epidemic and pandemic outbreaks occurred, coincident with an increase in research on the field. Today, we know the viral subtype

that caused the pandemic in 1918 (Reid *et al.*, 1999; Reid *et al.*, 2000), and the virus has been well characterized (Taubenberger *et al.*, 1997; Taubenberger *et al.*, 2005).

2.3. Influenza viruses

2.3.1. Classification and nomenclature

Influenza viruses A, B, and C, (IAV, IBV, and ICV, respectively) are included in the family *Orthomyxoviridae*, together with Thogotovirus and Isavirus. More recently, Quaranjavirus (Presti *et al.*, 2009) and Influenza Virus D (IDV) (Hause *et al.*, 2013) were included in this family.

Influenza A and B viruses cause seasonal epidemics yearly, presenting a social, health, and economical burden (Keech and Beardsworth; Nair *et al.*, 2011). Likewise, influenza A virus is also regarded as the only influenza virus with potential to cause pandemic outbreaks (Zambon, 2011).

Influenza A viruses are further divided into subtypes according to the two main surface glycoproteins: Hemagglutinin (HA; H for nomenclature) and Neuraminidase (NA; N for nomenclature). There are a total of 18 HA subtypes (H1 to H18) and 11 NA subtypes (N1 to N11) identified so far (Tong *et al.*, 2013; Saunders-Hastings and Krewski, 2016). The full name of a certain influenza virus must also include details such as the host of origin, the geographical origin, the strain number, and the year of isolation, following a naming convention (CDC, 2018). One example is "A/Perth/16/2009 (H3N2)" (in the case of human-origin viruses, the name of the host of origin is not given).

2.3.2. Influenza A virion structure and genomic organization

Influenza A virions have a size of approximately 100-200nm (Szewczyk *et al.*, 2014). The virion surface contains two glycoproteins, Hemagglutinin (HA) and Neuraminidase (NA), and an ion channel protein, Matrix Protein 2 (M2). These three proteins are embedded in the viral envelope,

which is obtained from the host cell membrane upon budding (Nayak *et al.*, 2009). Inside the envelope is Matrix Protein 1 (M1), which functions as a tether for the ribonucleoprotein (RNP) complex (Ruigrok *et al.*, 1989). RNP complex comprises the Nucleocapsid Protein (NP) and the 8 genomic fragments, associated with several copies of the viral polymerase proteins PA, PB1, and PB2 (Noda *et al.*, 2006) (Figure 1).

The 8 genomic fragments of Influenza A virus are negative single-stranded RNA, and code for the following proteins (ordered by length in base pairs): The first segment codes PB2; the second segment (PB1 segment) codes PB1 and the recently identified N40 and PB2-S1 (Wise *et al.*, 2009; Yamayoshi *et al.*, 2016); the third segment codes for PA; the fourth segment codes for HA; the fifth segment codes for NP; the sixth segment codes for NA; the seventh segment (M Segment) codes for M1 and M2; and the eight segment (NS) codes for Non-Structural Protein 1 (NS1) and Non-Structural Protein 2 (NS2/NEP).

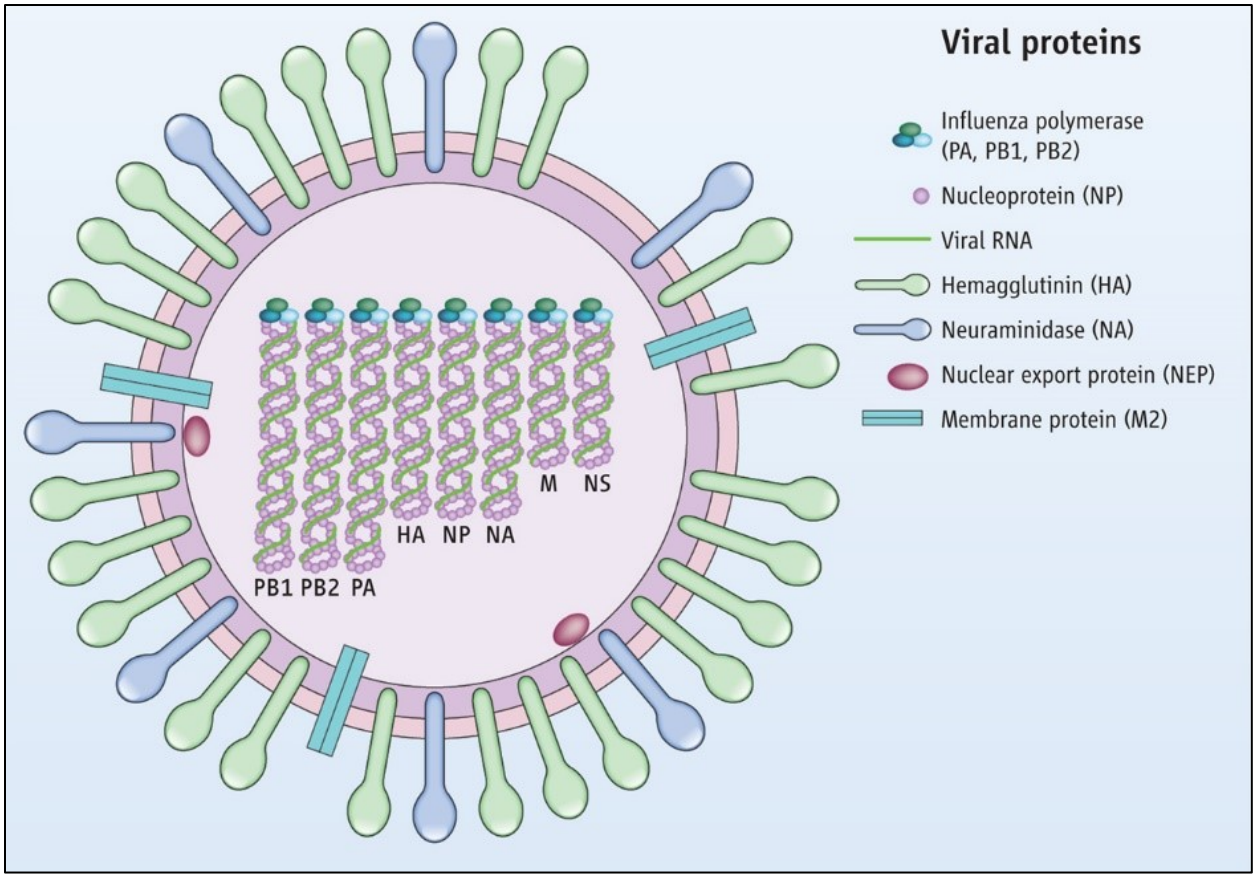

Figure 1. Schematic representation of Influenza A virus structure and genomic fragments

Influenza A virus contains two glycoproteins (hemagglutinin and neuraminidase) and an ion channel protein (M2) embedded on the viral envelope. Inside the envelope, M1 protein acts an anchor for the viral ribonucleoprotein complex, composed of eight single-stranded negative-sense RNA segments and eight nucleocapsid protein (NP) molecules, one associated to each of the RNA segments.

From Tao, Y. J. and W. Zheng (2012). "Visualizing the Influenza Genome." 338(6114): 1545-1546. Reprinted with permission from AAAS.

2.3.3. Influenza A replication cycle

The first step for Influenza A replication is binding of the virions to the cell receptor. This process is initiated by the HA1 subunit of HA that binds to receptors containing sialic acid chains (Matrosovich *et al.*, 2009). Structure and abundant of receptors, and structure and abundance of HA are factors that affect binding (Matrosovich *et al.*, 2006). Upon binding, receptor-mediated endocytosis occurs, through different possible mechanisms (Lakadamyali *et al.*, 2004), including clathrin-mediated endocytosis (Matlin *et al.*, 1981; Rust *et al.*, 2004), clathrin-independent endocytosis (Sieczkarski *et al.*, 2003; Rust *et al.*, 2004), and macropinocytosis (de Vries *et al.*, 2011).

After internalization into endosomes, the viral particles are directed towards the nucleus (Lakadamyali *et al.*, 2003) in an actin- and microtubules-dependent process (Sun and Whittaker, 2007; De Conto *et al.*, 2012). During this process, maturation and acidification of the endosome occurs; lowering of endosomal pH triggers a conformational change in HA, that leads to exposure of its HA2 subunit towards the endosomal membrane (Huotari and Helenius, 2011), which mediates fusion by pulling both viral and endosomal membranes together (Huang *et al.*, 2003; Garcia *et al.*, 2015). The acidification of the endosome also enables the M2 proton channel protein to open (Pinto *et al.*, 1992): as a result, the interior of the virion also becomes acid, which favors the dissociation of M1 from the viral RNP complex (Martin and Helenius, 1991) and causes the release of the latter into the cytosol (Zhirnov, 1990). NP protein mediates the nuclear transport of the RNP complex (Wu *et al.*, 2007).

Once in the nucleus, replication and transcription occur. For replication, the viral RNA (single strand, negative sense) is used to first synthesize a positive-sense complementary RNA (cRNA), that will be used as a template for new copies of viral RNA (Neumann *et al.*, 2004). This process

is mediated by both the viral RNA polymerase (Boivin *et al.*, 2010) and host cell factors (Nagata *et al.*, 2008).

The viral RNA is at the same time used for transcription, i.e., to obtain viral messenger RNA (mRNA). Viral polymerase proteins are able to bind to the host cell mRNAs (Blaas *et al.*, 1982), "steal" the host cell mRNAs 5' cap (Dias *et al.*, 2009), and use it as a primer for transcription of the viral mRNA (Braam *et al.*, 1983).

Some of the viral mRNAs are spliced in the nucleus to synthesize virus proteins that will drive the export of the viral products to the cytosol, such as M1 (Huang *et al.*, 2001) and NS2/NEP (O'Neill *et al.*, 1998).

Viral mRNAs of known viruses are translated in the cytoplasm using the host cell machinery (Panthu *et al.*, 2017). In the case of influenza, PABP1 and NS1 proteins associate with host cell translation factors to promote translation (Burgui *et al.*, 2003). HA and NA are glycosylated in the ER, key processes that determine the pathogenicity of influenza (Webster and Rott, 1987; Li *et al.*, 1993). Additionally, different viral proteins undergo other post-translational modifications (Matsuoka *et al.*, 2013).

HA and NA are directed towards lipid rafts (Kundu *et al.*, 1996; Takeda *et al.*, 2003). Here, vRNA segments are selectively recruited (Fujii *et al.*, 2003) and M2 protein regulates the budding of virions (Rossman *et al.*, 2010). NA facilitates release and prevents aggregation of the virions by removing sialic acids from cell receptors (Lamb and Krug, 1996; Wagner *et al.*, 2000) (Figure 2).

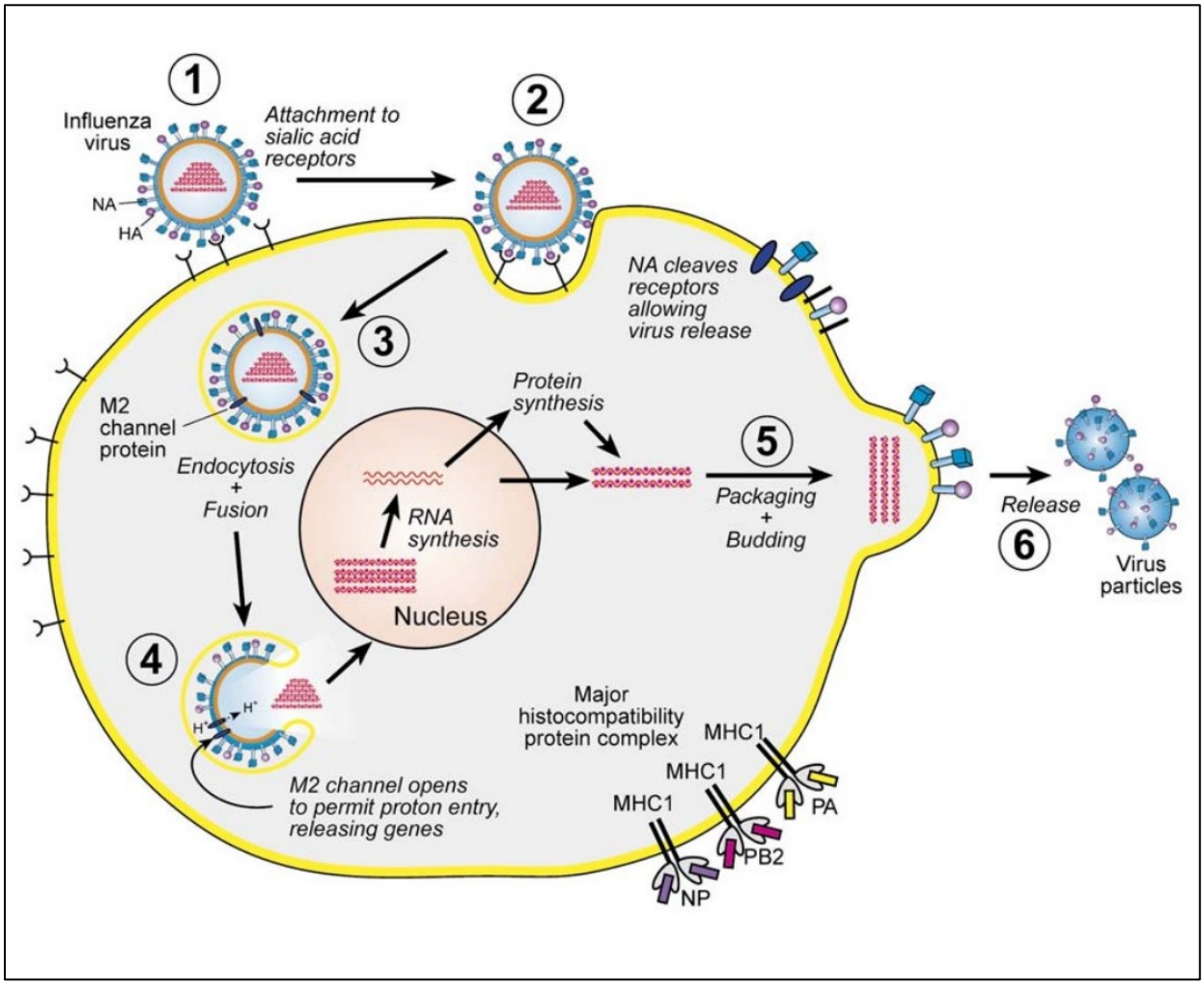

Figure 2. Life cycle of Influenza virus

Hemagglutinin (HA) proteins on the viral surface are responsible for binding to sialic acid-containing receptors on the cell membrane (1,2). The virion is typically internalized by receptor-mediated endocytosis (2,3). Acidification of the endosome leads to conformational changes in HA and opening of the M2 channels, resulting in fusion of viral/endosomal membranes (3,4). The fusion of these two membranes allows for the viral vRNPs to be released (4). vRNPs are directed to and transported into the nucleus in a process mediated by NP proteins (4). Viral replication and transcription occur in the nucleus. Spliced M1 and NEP mRNAs drive the export of viral mRNAs. Translation of these mRNAs and post-translation modifications occur in the cytoplasm. Proteins and vRNPs are directed towards the cytoplasm, and packaging occurs in proximity to lipid rafts (5). Release of the assembled virus occurs, facilitated by Neuraminidase (NA) (6).

Reprinted from Subbarao, K., et al. (2006). "Development of Effective Vaccines against Pandemic Influenza." Immunity 24(1): 5-9, with permission from Elsevier.

2.4. Pandemic influenza

2.4.1. Mechanisms for the generation of pandemic influenza

Influenza's fragmented genome makes possible genetic re-assortment, i.e., the exchange of genomic segments, when two different strains co-infect the same cell. This phenomenon is known as "antigenic shift": it accelerates viral evolution (Holmes *et al.*, 2005; Ince *et al.*, 2013), which results in the generation of different influenza strains (Reid and Taubenberger, 2003) and/or potential "novel" viruses (Vergara-Alert *et al.*, 2014). If the new variant achieved a major genetic change (Shao *et al.*, 2017) and is infectious and transmittable among humans (Saunders-Hastings and Krewski, 2016), the virus will have pandemic potential, since the population is not likely to have immunity. Although antigenic shift gives influenza potential for generating pandemic strains, there are other mechanisms such as replication errors or immune pressure that also contribute to pandemic influenza (Landolt and Olsen, 2007).

2.4.2. Impact of pandemic influenza

Since the "Spanish flu" in 1918 up until the present, there have been several pandemic events. The Spanish influenza was caused by an avian strain, H1N1, and killed approximately 50 million people world-wide (Johnson and Mueller, 2002), surprisingly including healthy young adults (Taubenberger and Morens, 2006; Taubenbergen and Morens, 2010). Interestingly, all Influenza A circulating today is derived from the H1N1 virus from 1918 (Taubenberger *et al.*, 2007).

Another pandemic event occurred during 1957 and 1958, named the "Asian influenza", and it was caused by H2N2 influenza, a descendant of the Spanish flu that had acquired new genomic segments (Scholtissek *et al.*, 1978). It originated in China, spread around the globe in a few months

(Dunn, 1958), and killed approximately 60.000 people in the United States alone (Henderson *et al.*, 2009).

The "Hong Kong influenza" pandemic followed in 1968-1969. Once again, re-assortment with avian influenza resulted in a new viral strain: H3N2 (Scholtissek *et al.*, 1978). Even though the virus spread very quickly (Cockburn *et al.*, 1969), the mortality associated with H3N2 influenza was much lower than the Spanish flu and even lower than the Asian flu (Morens *et al.*, 2009).

More recently, a pandemic outbreak occurred in 2009. A novel H1N1 virus appeared after re-assortment of previously circulating swine H1N2 and swine H1N1 strains (Garten *et al.*, 2009). This variant emerged in Mexico and the United states (Guan *et al.*, 2010). It affected at least 214 countries and it accounted for almost 20,000 deaths (WHO, 2010), although it was estimated to cause up to 500,000 (Dawood *et al.*, 2012), since laboratory-confirmed deaths are considered an under-estimate.

It is considered impossible to predict accurately the next pandemic strain (Saunders-Hastings and Krewski, 2016). Currently, the threat of pandemic influenza outbreaks resides in circulating strains such as H5N1 from avian reservoirs (Horimoto and Kawaoka, 2005), or H3N2, from swine reservoirs (Watson *et al.*, 2015).

2.5. Epidemic influenza

2.5.1. Mechanisms for the generation of epidemic influenza

It is generally accepted that RNA-dependent RNA polymerases lack proofreading capacity (Johnson *et al.*, 2017). In the case of Influenza A, this means a mutation rate of 7.3 mutations per 10^{-5} base pairs, or 1 mutation per genome replication (Drake, 1993) that can't be corrected for. Over time, this leads to an accumulation of mutations that primes antigenic variation, an effect

known as “antigenic drift” (Das et al 2013; Das *et al.*, 2013). When this variation affects proteins targeted by influenza vaccines, mainly the globular domain of HA (Virelizier, 1975; CDC, 2018), viruses containing these proteins will be able to escape immunity, giving them potential to cause epidemic outbreaks (van de Sandt *et al.*, 2012).

2.5.2. Impact of epidemic influenza

Factors that fluctuate seasonally such as humidity and temperature have been linked to the seasonality of influenza outbreaks (Deyle *et al.*, 2016); consequently, influenza activity is different in the northern and southern hemispheres (Cox and Subbarao, 2000). Currently, Influenza A(H1N1) (Hashem *et al.*, 2018), A(H3N2) and B (Skowronski *et al.*, 2018) strains are circulating among humans.

Seasonal influenza is responsible for millions of cases of severe illness and more than 500,000 deaths worldwide, every year (Rolfes *et al.*, 2018). Although influenza affects all age groups, it has been shown that mortality is disproportionately higher in children (Matias *et al.*, 2014) and elderly (Dao *et al.*, 2010). Altogether, this presents not only a public health issue but also an economic and social burden (Putri *et al.*, 2018).

	Estimated number of deaths (influenza)				% of total influenza	
	A/H1N1	A/H3N2	*B*	Total	Influenza A	Influenza B
1997/1998	1	17 351	149	17 500	99	1
1998/1999	5	17 481	6673	24 159	72	28
1999/2000	11	21 599	182	21 792	99	1
2000/2001	206	341	11 303	11 850	5	95
2001/2002	10	22 256	4696	26 962	83	17
2002/2003	148	2495	9376	12 020	22	78
2003/2004	0	28 821	424	29 245	99	1
2004/2005	1	17 126	7032	24 159	71	29
2005/2006	43	14 545	4869	19 458	75	25
2006/2007	293	5145	5610	11 048	49	51
2007/2008	90	14 745	11 312	26 148	57	43
2008/2009	125	632	4105	4862	16	84
Average season	78	13 545	5478	19 100	71	29

Figure 3. Mortality attributable to influenza by season between 1997 and 2009 in the US

The data presented is obtained from the number of samples tested positive for Influenza A/H1N1, Influenza A/H3N2, and Influenza B, reported by the Centers for Disease Control. For every season, data collected from the week 40 of the first year until the week 20 of the next year (both included).

Modified from Matias, G., et al. (2014). "Estimates of mortality attributable to influenza and RSV in the United States during 1997-2009 by influenza type or subtype, age, cause of death, and risk status." Influenza Other Respir Viruses 8(5): 507-515.

2.6. Prevention of Influenza

2.6.1. Vaccination

Edward Jenner is considered the father of vaccination. He observed, back in the 18th century, how farmers working with cows had reduced their vulnerability to smallpox outbreaks (Jenson *et al.*, 2016). Inferring that exposure to a similar disease offered protection against other infections, he took material from smallpox wounds initially, and later from cowpox, and inoculated other people with this material. Thus, he developed for the first time a vaccination method in humans (Greenwood, 2014).

Based on Jenner's discovery, Louis Pasteur designed a method for attenuation, in the 19th century. Vaccination was known to cause side effects, but Pasteur showed how exposing disease-causing organisms to adverse conditions would decrease these negative effects on patients (Plotkin and Plotkin, 2011). Jenner's and Pasteur's discoveries set the ground for future immunization/vaccination experiments in the following years.

Pasteur's attenuation of bacteria by exposure to heat to immunize individuals can be considered the first kind of vaccine ever obtained (attenuated vaccine) (Plotkin, 2014), although other scientists were able to improve this technique along the following years. In the decades of 1940 and 1950, it was discovered that passaging pathogens in anomalous hosts could also result in attenuation (Koprowski *et al.*, 1952). In vitro cell-culture was a later improvement, which allowed for future vaccines to be produced in sterile conditions and increased the efficiency of this method (Sabin *et al.*, 1954; Takanashi *et al.*, 1975).

By the end of the 19th century, experiments demonstrated that bacteria killed under certain conditions were able to maintain immunogenicity (Plotkin *et al.*, 2014). Applying this principle to

plague or typhoid bacteria resulted in the discovery of inactivated vaccines (Wright and Semple, 1897). Chemical inactivation was used in the 20th century for diphtheria treatment (Glenny and Hopkins, 1923) and, for the first time, for viral vaccines, such as the influenza vaccine (Francis and Magill, 1936).

Morphological and structural analyses of pathogens revealed that only some components of pathogens were responsible for immunogenicity; this idea is behind the creation of the first subunit vaccines (Moyle and Toth, 2013). This information was first used to design bacterial vaccines: capsular polysaccharides were used to prepare the meningococcal vaccine (Gotschlich *et al.*, 1969). Purified proteins appeared later as another type of subunit vaccine; such is the case of influenza hemagglutinin, used already in 1977 as a vaccination mechanism (Cate *et al.*, 1977).

Advances in research made it possible to genetically engineer organisms towards the end of the 20th century. In the context of vaccination, this permitted the arrival of recombinant vaccines, prepared first from hepatitis B antigens (Hilleman *et al.*, 1983). As technology moved forward, it became possible to tailor antigens to avoid the possible adverse effects that the native proteins could cause (Nascimento and Leite, 2012). Different methods allow for the construction not only of proteins and antigens but also whole organisms (Germanier and Füer, 1975) and virus-like particles (VLPs) (Kirnbauer *et al.*, 1992).

Vaccination is today defined as an active and artificial method of immunization (CDC, 2018): active because it stimulates the immune system to produce an immune response against a pathogen; and artificial as opposed to natural immunity, triggered by exposure of an organism to the pathogen. The objective of vaccination is inducing immunity minimizing or eliminating the disease caused by a given pathogen (Egli *et al.*, 2014). The most effective way to prevent infection by influenza is, not surprisingly, through vaccination (WHO, 2018).

2.6.2. The immune system

The immune system can be divided into two major branches: innate and adaptive.

The innate immune system is not antigen-specific and is present at birth (Georgountzou and Papadopoulos, 2017); anatomical and physiological barriers such as the skin or bacteriolytic enzymes can be considered as part of the innate immune system (Janeway and Medzhitov, 2002). Several cell types can also mediate or participate in innate immunity (Gasteiger *et al.*, 2017). The innate immune system acts as the first line of defense against pathogens, degrading the pathogens directly via secretion of enzymes or "defense" peptides (Linde *et al.*, 2008) or indirectly via phagocytosis or other intracellular mechanisms (Kennedy *et al.*, 2010). Despite lacking the ability to recognize pathogens in a specific manner, some cells in the innate immune system can recognize conserved molecular patterns in pathogens through receptors such as Toll-like receptors (Lee and Kim, 2007) or Nod-like receptors (Franchi *et al.*, 2009).

In contrast to the innate immune system, the adaptive immune system is acquired with development. This function is mainly carried out by lymphocytes (Kennedy *et al.*, 2010), which are able to recognize an antigen in a (more) specific manner (Vivier and Malissen, 2005), and present immunological memory (Crotty and Ahmed, 2004).

Lymphocytes can be classified into B lymphocytes, that develop and mature in the bone marrow (Eibel *et al.*, 2014); and T lymphocytes, that also originate in the bone marrow but differentiate and mature in the thymus (Koch and Radtke, 2011).

B cells or B lymphocytes express immunoglobulins in the form of B-cell receptor (BCR) (Ghia *et al.*, 1998) that can recognize antigens (Treanor, 2012). Upon BCR-antigen contact, activation of B cells (DeFranco, 1997) and differentiation into either plasma cells (Nutt *et al.*, 2015) or memory

B cells (Kurosaki *et al.*, 2015) occurs. Plasma cells mediate the humoral immune response, being responsible for antibody production (Fairfax *et al.*, 2008). Memory B lymphocytes are long-lived cells that activate quickly after a secondary antigen contact and have higher affinity for this antigen (Neuberger *et al.*, 2000).

In turn, the T-cell receptor (TCR) molecules expressed on the surface of T cells (or T lymphocytes) can only recognize antigen fragments (epitopes) presented on Major Histocompatibility Complex (MHC) molecules (Bjorkman *et al.*, 1987). Contact with these molecules activates the T cell (Manz *et al.*, 2011), which leads to cytokine production (Huang *et al.*, 2013). Exposure to antigen may also lead to formation of memory T cells that, in similarity with memory B cells, are able to rapidly respond in case of re-exposure to a given antigen (Kaech and Cui, 2012).

T cells can be subdivided in different groups depending on what cluster of differentiation (CD) molecules they express (or not) (Engel *et al.*, 2015). T cells expressing CD4 (CD4+) are called T "helper" because of their capacity to "help" B cells make antibodies, but they also coordinate the immune response through different other mechanisms (Zhu and Paul, 2008); CD4+ T cells play a crucial role in mediating (anti-) viral immunity (Sant and McMichael, 2012). CD8 positive (CD8+) T cells are known as "cytotoxic" because of their ability to quickly expand (Yoon *et al.*, 2010) and eliminate infected cells via their cytotoxic capacity (Callan *et al.*, 2000); CD8+ T cells have also been shown to participate in and control viral infections (Gulzar and Copeland, 2004).

2.6.3. Antigen presenting cells and antigen presentation

Cells such as dendritic cells (DCs) (Thery and Amigorena, 2001) and macrophages (Unanue, 1984) are able to contact an antigen, load it on MHC molecules, and ultimately present it to T lymphocytes to mediate their activation: these cells are known as antigen presenting cells (APCs). Antigen presenting cells are, in fact, the only cells that express MHC II molecules (Trombetta and Mellman, 2005).

Antigens to be presented on MHC molecules enter the antigen presenting cells via different mechanisms (Figure 4). Clathrin-mediated endocytosis (CME) involves the internalization of particles bound to a cell-receptor in clathrin-coated vesicles that invaginate from the plasma membrane (Schmid and McMahon, 2007). Alternatively, there are other machineries for endocytosis of particles that don't require clathrin (Doherty and McMahon, 2009): they can be referred to as clathrin-independent endocytosis (CIE) mechanisms. Caveolin-dependent endocytosis involves formation of caveolin-coated vesicles or caveolae, morphologically distinguishable from clathrin-coated vesicles (Richter *et al.*, 2008). Macropinocytosis is another example of clathrin-independent endocytosis that involves projections of the plasma membrane that engulf extracellular material (Swanson and Watts, 1995). In the context of antigen presentation, the very mechanisms of internalization of an antigen can pre-determine the MHC molecules they will be loaded on. For example, CME and macropinocytosis can favour the pathways that lead to MHC II presentation (Blum *et al.*, 2013); in contrast, caveolin-dependent endocytosis can facilitate MHC I presentation (Jancic *et al.*, 2007).

The endocytic vesicles formed by the mentioned mechanisms typically recruit Rab5 molecules that will later interact with EEA1 molecules present in the early endosome: this interaction will result in the fusion of the endocytic vesicles with the early endosome (Selby *et al.*, 2017). The

early endosome is an organelle in charge of separating those protein products into degradative or recycling compartments (Piper and Katzmann, 2007).

The degradative pathway is followed when the early endosome starts acidifying after fusion with endocytic vesicles and continues to do so as it matures to become the late endosome, where the pH reaches 5.5 (Casey *et al.*, 2010). The late endosome can fuse with the lysosome, where the pH becomes 4.7, and different acid hydrolases favor the breakdown of the material contained in them (Lübke *et al.*, 2009). MHC II molecules contained in the lysosomes can now incorporate fragmented antigens. These peptide-loaded MHC II (pMHC II) molecules are expressed and presented on the plasma membrane (Blum *et al.*, 2013), which will lead to activation of CD4+ T cells in the lymph nodes (Itano and Jenkins, 2003). Additionally, antigen presenting cells express MHC I molecules, as in many other cell types (Greene *et al.*, 2017).

In contrast, the loading of exogenous antigens on MHC I by antigen presenting cells occurs through cross-presentation, typically in recycling endosomes (Guermonprez *et al.*, 2002). Different mechanisms and organelles have been proposed to be involved in this process that do not involve endosomal maturation or lysosomal fusion (Cruz *et al.*, 2017). Peptide-loaded MHC I molecules are presented on the membrane and ultimately interact with CD8+ T lymphocytes, which results in the latter's activation (Zinkernagel and Doherty, 1974; Zhang and Bevan, 2011).

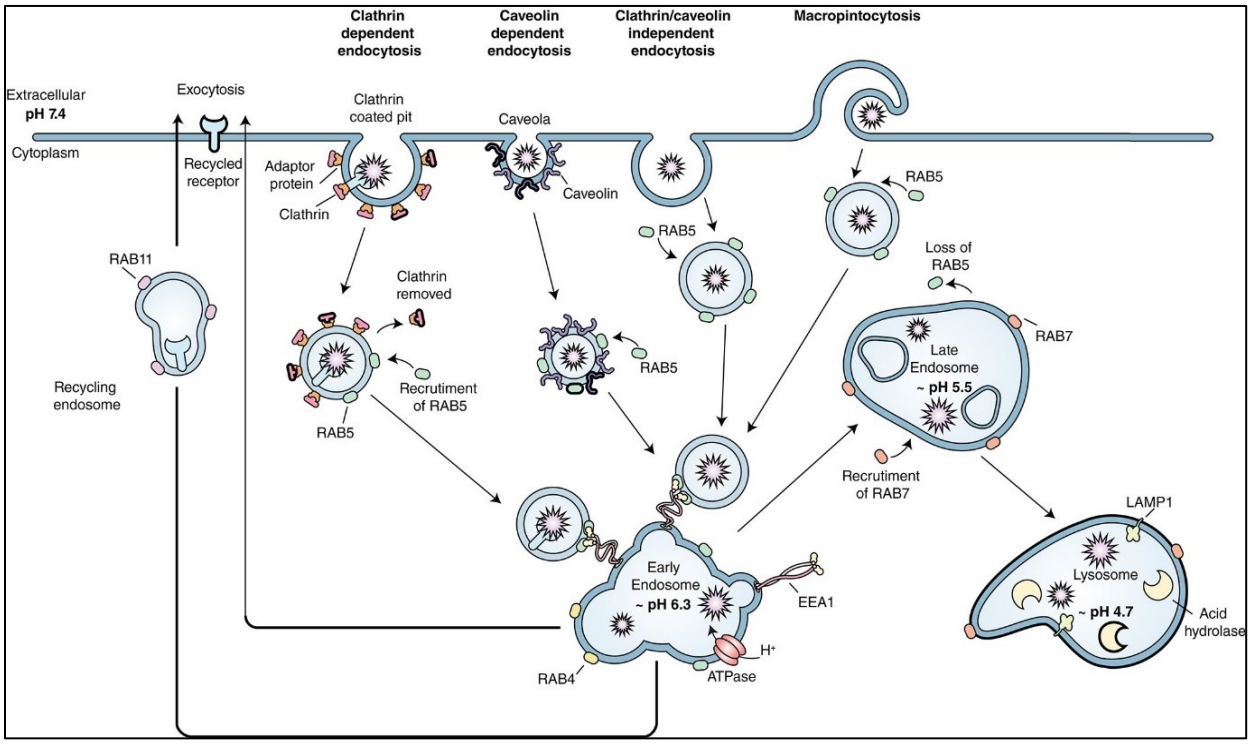

Figure 4. Schematic overview of internalization mechanisms and endosomal trafficking

Antigens can enter the cell via different mechanisms, including clathrin-coated vesicles, caveolin-coated vesicles, or vesicles formed by macropinocytosis. These vesicles recruit Rab5 that will mediate their attachment to EEA1, present in the early endosome, resulting in their fusion. The cargo can be then delivered back to the membrane via recycling endosomes or undergo acidification. Acidification and recruitment of Rab7 to the early endosome will result in the formation of the late endosome, where the pH reaches 5.5. The late endosome may bind to the lysosome, where the lower pH (4.7) will favor protein degradation.

2.6.4. Vaccines against influenza

Currently, there are 3 different forms of influenza vaccines available (CDC, 2018; Figure 5): live attenuated (LAIV), inactivated (IIV) and recombinant (RIV) influenza vaccines. These three types of vaccines are also classified in trivalent (TIV) or quadrivalent (QIV), depending on whether they target three or four influenza strains.

Live attenuated influenza vaccines are based on introducing mutations that affect the viral development or pathogenicity (Coelingh *et al.*, 2014). LAIVs can induce both humoral (antibody production) and cell-mediated (not involving antibody production) immune responses (Hoft *et al.*, 2017). The Flumist vaccine produced by Medimmune Vaccines was the only licensed LAIV for the 2017/2018 season, however, the use for that season was not recommended in the US (CDC, 2018).

Inactivated influenza vaccines are obtained in different forms (whole inactivated vaccines, split virus vaccines, subunit vaccines, and virosomal vaccines) (Soema *et al.*, 2015). Most commonly used IIVs are either split vaccines, obtained by fragmentation of their membrane (Neurath *et al.*, 1971); or subunit vaccines, produced by separation of viral surface proteins from the nucleocapsid (Laver and Webster, 1976). Immune response induced by IIVs varies depending on age groups, but they are able to induce antibody production (Houser and Subbarao, 2015) and, in adults, also a modest cell-mediated immune response (Hoft *et al.*, 2017). There are different IIVs licensed, including trivalent and quadrivalent, and in different formulations (CDC, 2018).

Recombinant influenza vaccines are made through expression of viral proteins in mammalian (Powers *et al.*, 1995) or insect (Treanor *et al.*, 2006) cells. These vaccines can be constructed in different ways, including virus-like particles (VLPs) (Sedova *et al.*, 2012). The only RIVs licensed

are Flublok trivalent and quadrivalent vaccines (Protein Sciences Corporation) (CDC, 2018): Flublok have been shown to induce strong antibody production and protection against influenza (Treanor *et al.*, 2007).

Trade name	Manufacturer	Presentation	Age indication	Mercury (from thimerosal, µg/0.5 mL)	Latex	Route
Inactivated influenza vaccines, quadrivalent (IIV4s), standard-dose†						
Afluria Quadrivalent	Seqirus	0.5 mL prefilled syringe	≥18 years	NR	No	IM§
		5.0 mL multidose vial	≥18 years (by needle/syringe) 18 through 64 years (by jet injector)	24.5	No	IM
Fluarix Quadrivalent	GlaxoSmithKline	0.5 mL prefilled syringe	≥3 years	NR	No	IM
FluLaval Quadrivalent	ID Biomedical Corp. of Quebec (distributed by GlaxoSmithKline)	0.5 mL prefilled syringe	≥6 months	NR	No	IM
		5.0 mL multidose vial	≥6 months	<25	No	IM
Fluzone Quadrivalent	Sanofi Pasteur	0.25 mL prefilled syringe	6 through 35 months	NR	No	IM
		0.5 mL prefilled syringe	≥3 years	NR	No	IM
		0.5 mL single-dose vial	≥3 years	NR	No	IM
		5.0 mL multidose vial	≥6 months	25	No	IM
Inactivated influenza vaccine, quadrivalent (ccIIV4), standard-dose,† cell culture-based						
Flucelvax Quadrivalent	Seqirus	0.5 mL prefilled syringe	≥4 years	NR	No	IM
		5.0 mL multidose vial	≥4 years	25	No	IM
Inactivated influenza vaccine, quadrivalent (IIV4), standard-dose, intradermal¶						
Fluzone Intradermal Quadrivalent	Sanofi Pasteur	0.1 mL single-dose prefilled microinjection system	18 through 64 years	NR	No	ID**
Inactivated Influenza Vaccines, trivalent (IIV3s), standard-dose†						
Afluria	Seqirus	0.5 mL prefilled syringe	≥5 years	NR	No	IM
		5.0 mL multidose vial	≥5 years (by needle/syringe) 18 through 64 years (by jet injector)	24.5	No	IM
Fluvirin	Seqirus	0.5 mL prefilled syringe	≥4 years	≤1	Yes††	IM
		5.0 mL multidose vial	≥4 years	25	No	IM
Adjuvanted inactivated influenza vaccine, trivalent (aIIV3),† standard-dose						
Fluad	Seqirus	0.5 mL prefilled syringe	≥65 years	NR	Yes††	IM
Inactivated Influenza Vaccine, trivalent (IIV3), high-dose§§						
Fluzone High-Dose	Sanofi Pasteur	0.5 mL prefilled syringe	≥65 years	NR	No	IM
Recombinant Influenza Vaccine, quadrivalent (RIV4)¶¶						
Flublok Quadrivalent	Protein Sciences	0.5 mL prefilled syringe	≥18 years	NR	No	IM
Recombinant Influenza Vaccine, trivalent (RIV3)¶¶						
Flublok	Protein Sciences	0.5 mL single-dose vial	≥18 years	NR	No	IM
Live Attenuated Influenza Vaccine, quadrivalent (LAIV4)* (not recommended for use during the 2017–18 season)**						
FluMist Quadrivalent	MedImmune	0.2 mL single-dose prefilled intranasal sprayer	2 through 49 years	NR	No	NAS

Abbreviations: ACIP = Advisory Committee on Immunization Practices; ID = intradermal; IM = intramuscular; NAS = intranasal; NR = not relevant (does not contain thimerosal).

* Immunization providers should check Food and Drug Administration–approved prescribing information for 2017–18 influenza vaccines for the most complete and updated information, including (but not limited to) indications, contraindications, warnings, and precautions. Package inserts for U.S.-licensed vaccines are available at https://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm093833.htm. Availability of specific products and presentations might change and differ from what is described in this table and in the text of this report.

† Standard dose intramuscular IIVs contain 15 µg of each vaccine HA antigen (45 µg total for trivalents and 60 µg total for quadrivalents) per 0.5 mL dose.

§ For adults and older children, the recommended site for intramuscular influenza vaccination is the deltoid muscle. The preferred site for infants and young children is the anterolateral aspect of the thigh. Specific guidance regarding site and needle length for intramuscular administration is available in the ACIP General Best Practice Guidelines for Immunization, available at https://www.cdc.gov/vaccines/hcp/acip-recs/general-recs/index.html.

¶ Quadrivalent inactivated influenza vaccine, intradermal: a 0.1-mL dose contains 9 µg of each vaccine HA antigen (36 µg total).

** The preferred injection site is over the deltoid muscle. Fluzone Intradermal Quadrivalent is administered per manufacturer's instructions using the delivery system included with the vaccine.

†† Syringe tip cap might contain natural rubber latex.

§§ High-dose IIV3 contains 60 µg of each vaccine antigen (180 µg total) per 0.5 mL dose.

¶¶ RIV contains 45 µg of each vaccine HA antigen (135 µg total for trivalent 180 µg total for quadrivalent) per 0.5 mL dose.

*** ACIP recommends that FluMist Quadrivalent (LAIV4) not be used during the 2017–18 season.

Figure 5. Influenza vaccines recommendation for the season 2017-2018 in the United States

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2.6.5. Limitations of influenza vaccines and potential solutions

Despite the availability of diverse types of influenza vaccines, vaccine efficacy (VE) varies yearly (Ortiz *et al.*, 2013). This is because the different vaccines present limitations (Cohen, 2017). Vaccine efficacy is especially low in the elderly due to "immuno-senescence", or a gradual loss in immune system efficacy as the age of an individual increases (Haq and McElhaney, 2014).

Without regard to the platform in which they are made, most influenza vaccines are made by growing the virus in chicken eggs (Rajao and Perez, 2018). Limitations to this approach are a lengthy time of vaccine production (Gerdil, 2003), and the implicit necessity of chicken egg supply (Soema *et al.*, 2015): these characteristics together with egg adaptations and mutations derived from the egg-based production are often associated with antigenic mismatch between the end vaccine product and the circulating strains of influenza (Paules *et al.*, 2018), which results in low vaccine efficacy.

On the other hand, the antigenic drift that influenza undergoes means that vaccines need to be re-formulated seasonally to match the possible novel circulating viruses (Houser and Subbarao, 2015).

To overcome these disadvantages of the currently licensed vaccines for influenza, research is being conducted on the production of "universal vaccines", targeting conserved epitopes of influenza (Berlanda Scorza *et al.*, 2016), that would avoid the necessity of annual re-vaccination and would potentially offer protection in case of a pandemic outbreak (Subbarao and Matsuoka, 2013).

In order to elude the dependence on chicken egg supplies, several recombinant vaccines; DNA vaccines, based on the expression of DNA constructs on the host cell (Yin *et al.*, 2009); and virus-

like particles (VLPs), particles containing viral antigens but lacking genetic material (Giles and Ross, 2011), are being researched.

2.6.6. Medicago's Virus-Like Particles (VLPs)

One promising novel approach in influenza vaccination are the Virus-Like Particles developed by Medicago Inc. These VLPs are obtained by transient expression of influenza hemagglutinin in *Nicotiana benthamiana* plant leaves, using *Agrobacterium* as a vector (D'Aoust *et al.*, 2008; D'Aoust *et al.*, 2010).

These VLPs have been recently shown to recapitulate features of virus-cell interactions. Their structure is similar to that of the influenza virus (Lindsay *et al.*, 2018), and there are also able to interact with and stimulate immune cells (Hendin *et al.*, 2017; Lindsay *et al.*, 2018), in a sialic acid-dependent manner (Makarkov *et al.*, 2017). Furthermore, Medicago's VLPs elicit strong and cross-reactive cell-mediated immune response and antibody production in mice (D'Aoust *et al.*, 2008; Pillet *et al.*, 2015) and in humans (Landry *et al.*, 2014; Pillet *et al.*, 2016). However, the underlying intracellular mechanisms that determine these vaccines' immunogenicity are yet to be completely understood.

3. OBJECTIVES

As mentioned above, the hemagglutinin-bearing VLPs developed by Medicago recapitulate wild-type influenza virus interactions with immune cells and elicit strong humoral and cell-mediated immune responses. This approach, independent of egg-based production, in combination with short production times in the manufacture of this particles and the cost-efficiency of the process, confers potential advantages to Medicago's vaccine over other products that take longer to produce and focus on antibody production and do not elicit such a strong and cross-reactive immune response. However, some aspects regarding the biology of Medicago's virus-like particles have still not been elucidated.

In this work, we aimed to study the interactions of the VLPs using human antigen presenting cells, and to understand how these interactions may affect or determine the unique immune response generated by the particles. In concrete, we used hemagglutinin 1 (H1) VLPs as a vaccine and human monocyte-derived macrophage (hMDMs) as an antigen presenting cell model. Considering this, the specific objectives set for this project were:

a) To understand what cellular processes are involved in the internalization of H1 VLPs into hMDMs and how this may be related to the unique immunogenicity profile generated by the particles.
b) To understand how, after internalization, are the H1 VLPs handled by the hMDMs, and how the intracellular processing of these particles may determine their immunogenicity.
c) To compare the results obtained when using H1 VLPs to those obtained when using soluble H1 protein as a comparator, which is commercially available, and how the differences might explain the potential advantages of the VLPs over other vaccine products.

4. MATERIALS AND METHODS

4.1. Influenza hemagglutinin (H1)-bearing plant-derived Virus-Like Particles (H1 VLPs) and recombinant H1 protein (soluble HA)

The virus-like particles used in this study were manufactured by Medicago Inc (Quebec, QC) through transient expression of influenza hemagglutinin in *N. benthamiana* plant leaves, as described previously (D'Aoust et al., 2010). The sequence of influenza HA was based on the sequence of Influenza A/California/07/2009 H1N1, rendering H1 VLPs. The HA content of these VLPs was 927μg/ml.

In some experiments, recombinant H1 protein (soluble HA protein) was used. It was produced in embryonic kidney 293 cells by Immune Technology (New York, NY). The sequence of HA was also based on Influenza A/California/07/2009 H1N1. The HA content of the stock solution of soluble H1 was 100μg/100μl (1,000 μg/ml).

4.2. Human monocyte-derived macrophages (hMDMs)

Peripheral blood was drawn from healthy donors between the ages of 20-50 that had provided written informed consent, and with the approval from the Research Ethics Committee of McGill University Health Centre (MUHC). Peripheral blood mononuclear cells (PBMCs) were first separated from the blood by centrifugation in SepMate-50 tubes from STEMCELL (Vancouver, BC). Monocytes were separated using magnetic beads (EasySep Human Monocyte Enrichment kit) from STEMCELL, based on negative selection. Monocytes were plated on 8-chamber cell imaging coverglasses (Eppendorf, Hamburg, Germany) at a number of 500,000 cells per chamber (applied 500μl of cells solution at 1,000,000 cells/ml). These monocytes were cultured in differentiation medium. This medium contained RPMI-1640 with 50 IU/ml of penicillin, 50μg/ml

of streptomycin, and 10mM HEPES (all reagents from Wisent, Saint-Jean Baptiste, QC). Additionally, 10% FBS (Wisent) and 20ng/ml of macrophage colony-stimulating factor (Gibco, Frederick, MD) were added to stimulate the differentiation of macrophages. Cells were kept in an incubator at 37°C and 5% CO_2 for a total of 7 days. At days 3 and 6, the differentiation medium was partially replaced by freshly prepared medium (supplemented with FBS and colony-stimulating factor).

4.3. Assessment of endocytic vesicles and endosomal structures formation/stimulation induced by H1 VLPs in hMDMs

hMDMs were incubated with H1 VLPs or soluble HA protein for 5 min, 15 min, 45 min (15 min incubation with H1 VLPs or soluble HA protein followed by 30 min incubation with growth medium), and 2 hours with H1 VLPs (15 min incubation with H1 VLPs followed by 105 min incubation with growth medium); hMDMs unexposed to HA served as control. Samples were kept in an incubator at 37°C and 5% CO_2 during the incubation times indicated. After this, cells were fixed with 2.5% glutaraldehyde (EMS Inc., Hatfield, PA) at 37°C for 15 min initially, and then slowly cooled down to 4°C over 30 min. Glutaraldehyde was replaced, and samples kept at 4°C overnight.

The day after, resin embedding for electron microscopy was performed. Samples were initially washed with 0.1M sodium cacodylate (EMS Inc.) three times for 20 min each. Post-fixation with 1% osmium tetroxide (EMS Inc.) in potassium ferrocyanide (Fisher Scientific, Pittsburgh, PA) for one hour at 4°C followed. Samples were then washed for 10 min three times with distilled water. Partial dehydration with ethanol solutions at 30%, 50%, and 70% for 8 min each were done before *en bloc* staining with 2% uranyl acetate for 45 min at 4°C. The dehydration was then completed by using ethanol at 80% and 90% for 8 min each, plus 100% ethanol three times for 10 min. The

cells were embedded in EPON812 resin (EMS Inc.) progressively: a mixture of 1:1 of EPON812:100% Ethanol was first added for 30 min, followed by a mixture 3:1 of the same reagents for another 30min. Pure EPON812 was added for 1 hour, and then once again for new EPON812. Samples were introduced in the oven at 57°C for at least 48 hours for the resin to polymerize.

Once resin was polymerized, the different conditions were sectioned using a Reichert-Jung Ultracut E microtome (Leica Microsystems Inc., Concord, ON) equipped with a Diatome 3.00 mm 45 Degree Ultrathin diamond knife (Edge Scientific Inc., Woodlawn, ON). Ultrathin sections were collected on Carbon Type-B Triple Slot, Cu grids (Ted Pella Inc., Redding, CA) and posteriorly stained with 4% Uranyl Acetate for 8 min and lead citrate for 5 min.

A Tecnai T12 microscope (Thermo Fisher Scientific, Rockford, IL) equipped with an AMT XR80C CCD camera (Advanced Microscopy Techniques Corp., Woburn, MA) was used for electron microscopy imaging. Micrographs at different magnifications were collected from the sections to assess endocytic vesicles formation and endosomal structures formation.

4.4. Identification of internalization mechanisms of H1 VLPs in hMDMs using endocytosis inhibitors

Several endocytosis inhibitors were used to evaluate their effects in the internalization of H1 VLPs by hMDMs. Chlorpromazine has been suggested to deplete clathrin from the cell membrane and thus inhibit clathrin-coated vesicle formation, or Clathrin-Mediated Endocytosis (CME) (Wang *et al.*, 1993; Daniel *et al.*, 2015). Caveolin-coated vesicles require the phosphorylation of caveolin-1 to pinch off from the plasma membrane (Tiruppathi *et al.*, 1997); Genistein blocks this mechanism through its tyrosine-kinase inhibiting activity (Akiyama *et al.*, 1987). Amiloride prevents

Rac1/Cdc42 signaling, disrupting acting remodeling that is ultimately required for macropinocytosis to occur (Koivusalo *et al.*, 2010). Dynasore disrupts the activity of dynamin (Macia *et al.*, 2006), so it has been proposed to inhibit both CME and Clathrin-Independent Endocytosis (CIE), and possibly macropinocytosis (Preta *et al.*, 2015).

Endocytosis inhibitors were applied in RPMI-1640 medium with penicillin, streptomycin, and HEPES at the following concentrations: chlorpromazine hydrochloride at 10 μg/ml, genistein at 200 μM, amiloride hydrochloride at 1 mM, and dynasore hydrate at 50 μM (all from Sigma-Aldrich, St. Louis, MO).

Subsequently, the solutions of H1 VLPs (at a final concentration of 15μg/ml based on HA content) with the different inhibitors at the final concentration indicated above or the H1 VLPs alone (with no inhibitors, as a control) were added, for a total of 5 min, 15 min, or 45 min (15 min of incubation with H1 VLPs/inhibitor followed by 30 min incubation with growth medium). Additional controls with no H1 VLPs added were included for the purpose of background immunofluorescence intensity determination. For the duration of this experiment, the samples were kept in an incubator at 37°C and 5% CO_2.

Samples were post-fixed with 2% methanol-free formaldehyde (Thermo Fisher Scientific) for 15 min at room temperature.

For the experiments where permeabilization took place, samples were permeabilized and blocked for 5 min at 4°C with 0.2% Triton X-100 (Sigma-Aldrich) plus goat normal serum 1% (EMD Millipore Corporation, Darmstadt, Germany). When permeabilization did not take place, samples were blocked with goat normal serum alone (1% in PBS).

Mouse monoclonal primary anti-H1 antibody, clone IVC102 (Meridian Life Science, Memphis, TN), was applied overnight at 4°C. Primary antibody was diluted in PBS. Secondary antibody Alexa Fluor ® 647, Fluoronanogold ™ goat anti-mouse IgG (Nanoprobes, Yaphank, NY) was applied for an hour, at room temperature. Secondary antibody was diluted in 1% milk in PBS. The dilution of the antibodies is indicated in Table 2.1. A drop (per condition) of NucBlue Live ReadyProbes Reagent-4'6-diamino-2-phenylindole (DAPI) (Thermo Fisher Scientific) was added to stain cell nuclei, prior to imaging.

Imaging was done using a Zeiss LSM780 confocal microscope equipped with a 100x/1.40 oil DIC Plan-Apochromat objective at the RI-MUHC Molecular Imaging Core facility (Montreal, QC).

Immediately after imaging, samples were fixed with 2.5% glutaraldehyde (EMS Inc.) at room temperature for 30 min. Glutaraldehyde was then replaced, and samples were kept at 4°C overnight.

A 0.1 M sodium cacodylate solution (EMS Inc.) was used to wash the samples, 3 times for 20 min, and then the HQ Silver enhancement kit (Nanoprobes) was used for 30 sec. Post-fixation in osmium tetroxide, washing, dehydration, embedding in resin, sectioning, and imaging were performed as indicated in the previous section (resin embedding for electron microscopy and electron microscopy imaging).

4.5. Identification of endosomal processing mechanisms for antigen presentation of H1 VLPs in hMDMs using common organelle markers

To follow the endosomal pathway of hemagglutinin after H1 VLPs were internalized, the following organelle markers were used for the purpose of co-localization analysis: EEA1 is present in the early endosomes and promotes the fusion of Rab5-containing vesicles, coming from the cell

membrane (Selby *et al.*, 2017); clathrin may remain in endocytic vesicles after fusing to the early endosomes; clathrin-rich regions of the early endosomes might be involved in protein sorting towards the lysosome (Raposo *et al.*, 2001); Rab4 is present in recycling vesicles coming from the early endosome towards the plasma membrane (Mohrmann *et al.*, 2002); Rab3c is localized to other recycling endosomal compartments, and has been shown to co-localize with MHC I (Zou *et al.*, 2009); LAMP1 is one of the major components of the lysosomal membranes, having a structural role in these organelles (Eskelinen, 2006).

hMDMs were incubated with H1 VLPs or soluble HA in medium at 15 μg/ml (by HA content) for either 15 min or 45 min (15 min incubation with H1 VLPs or soluble H1 followed by 30 min of incubation with medium). Samples were in the incubator at 37°C and 5% CO_2 for the length of the experiment.

Samples were post-fixed with 4% methanol-free formaldehyde for 15 min at room temperature and permeabilized and blocked for 1 hour with 0.3% Triton X-100 (Sigma-Aldrich) plus 5% goat and 5% donkey serum (EMD).

Immuno-staining of hemagglutinin was done using mouse Mab to Influenza A H1 clone IVC102 (Meridian Life Science, Memphis, TN). Rabbit anti-EEA1 and anti-clathrin (both from Cell Signaling Technology, Danvers, MA), anti-Rab4 and anti-Rab3c (both from Thermo Fisher Scientific), and anti-LAMP1 (Abcam Inc, Toronto, ON) antibodies were used to label different endosomal structures. All these primary antibodies were applied overnight at 4°C. Secondary antibodies Alexa Fluor ® 647-conjugated goat anti-rabbit IgG and Alexa Fluor ® 488-conjugated donkey anti-mouse IgG (both from Thermo Fisher Scientific) were added for an hour, and samples left at room temperature. All primary and secondary antibodies were applied in 5% goat plus 5% donkey serum containing 0.3% Triton X-100 at the dilutions indicated on Table 2.1. A drop (per

condition) of NucBlue Live ReadyProbes Reagent-4'6-diamino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Rockford, IL) was added before to imaging. Imaging was performed as previously described.

Antibody	Dilution/Concentration
Mouse anti Influenza A H1	1:200
Rabbit anti-EEA1	1:100
Rabbit anti-clathrin	1:50
Rabbit anti-Rab4	1:250
Rabbit anti-Rab3c	1:100
Rabbit anti-LAMP1	1:200
Alexa Fluor ® 647, Fluoronanogold ™ goat anti-mouse	2.0 μg/mL
Alexa Fluor ® 647, goat anti-rabbit	1:1000
Alexa Fluor ® 488, donkey anti-mouse	1:1000

Table 1. Primary and secondary antibodies dilutions

4.6. Image analyses

For endocytosis inhibition assays, background intensity of fluorescence was first calculated and then subtracted from every condition's intensity of fluorescence, to obtain a corrected intensity of fluorescence value. This was done as follows:

First, average background intensity of fluorescence was calculating by averaging the fluorescence intensity of control cells (where H1 VLPs had not been applied). Using ImageJ, one Region Of Interest (ROI) per cell was drawn manually, then intensity of fluorescence calculated. The intensities obtained from all the cells in this control were averages and used as the average background intensity of fluorescence.

For each other condition, ImageJ was used to draw ROIs around individual cells and then intensity of fluorescence was calculated (per cell). From every individual intensity of fluorescence value (for the individual ROIs), the average background intensity of fluorescence (previously calculated) was subtracted, obtaining a corrected intensity of fluorescence value. For each time point and inhibitor applied, the average values of corrected intensity of fluorescence values were calculated.

For co-localization experiments, Pearson's correlation coefficient and Pearson's correlation coefficient above threshold were obtained per condition, as follows:

For every time condition, ImageJ was used to draw ROIs manually, surrounding individual cells. PureDenoise plugin on ImageJ was used to subtract noise from all the channels, and then ImageJ was used to subtract background on the de-noised channels. Finally, ImageJ co-localization threshold analysis was performed for the de-noised and background-free ROIs, to obtain individual correlation coefficients values.

4.7. Statistical analyses

For all statistical analyses shown, One-Way analysis of variance (One-Way ANOVA) with multiple comparisons or unpaired t test were calculated using GraphPad Prism 6.0 software. Data from 2 or more experiments were analyzed.

5. RESULTS

5.1. H1 VLPs stimulate endocytic vesicles formation in hMDMs

To understand the route that the VLPs follow for internalization, we observed hMDMs exposed to H1 VLPs and compared them to non-exposed cells, using electron microscopy. The resolution obtained with this technique is sufficient to morphologically differentiate endocytic vesicles directly (Figures 6A, 6B, 6C). Based on their morphological appearance and size (100-200nm), a subclass of endocytic vesicles, namely clathrin-coated vesicles, can be distinguished by their bristle-like protein coating (Roth and Porter, 1964) (Figure 6D). Consequently, a sub-classification of endocytic vesicles can be made into clathrin-coated vesicles, or uncoated vesicles, which lack that characteristic coating (Figure 6E). The uncoated vesicles visualized by electron microscopy possibly include caveolin-coated vesicles (or caveolae), according to their morphology and size range (50-100nm in diameter) (McIntosh *et al.*, 2001).

Quantification of the number of endocytic vesicles present in hMDMs after being incubated with H1 VLPs or soluble HA for different time points was made (Figures 6F and 6G), in order to determine whether they were able to stimulate the formation of such vesicles.

The number of endocytic vesicles increased significantly in hMDMs incubated with H1 VLPs for 15 minutes. The increase was highly significant at 45 minutes of incubation and decreased close to initial (control) values at 2 hours: similarly, the individual pools of clathrin-coated vesicles and uncoated vesicles counted separately also increased significantly at 15 minutes and 45 minutes and decreased at 2 hours (Figure 6F).

In turn, when hMDMs were incubated with the soluble HA protein, neither the number of endocytic vesicles in total nor the number of vesicles counted individually showed a significant increase, at any incubation times (Figure 6G).

In summary, there was an increase in both clathrin-coated and uncoated endocytic vesicles in hMDMs incubated with H1 VLPs for 15 min and for 45 min. Such an increase was not observed when the cells were incubated with soluble HA at any of the time points studied. These observations suggest that H1 VLPs may be efficiently internalized by at least two different endocytic processes, involving the formation of clathrin-coated vesicles and of "uncoated" (lacking clathrin coat) vesicles, possibly including caveolin-coated vesicles.

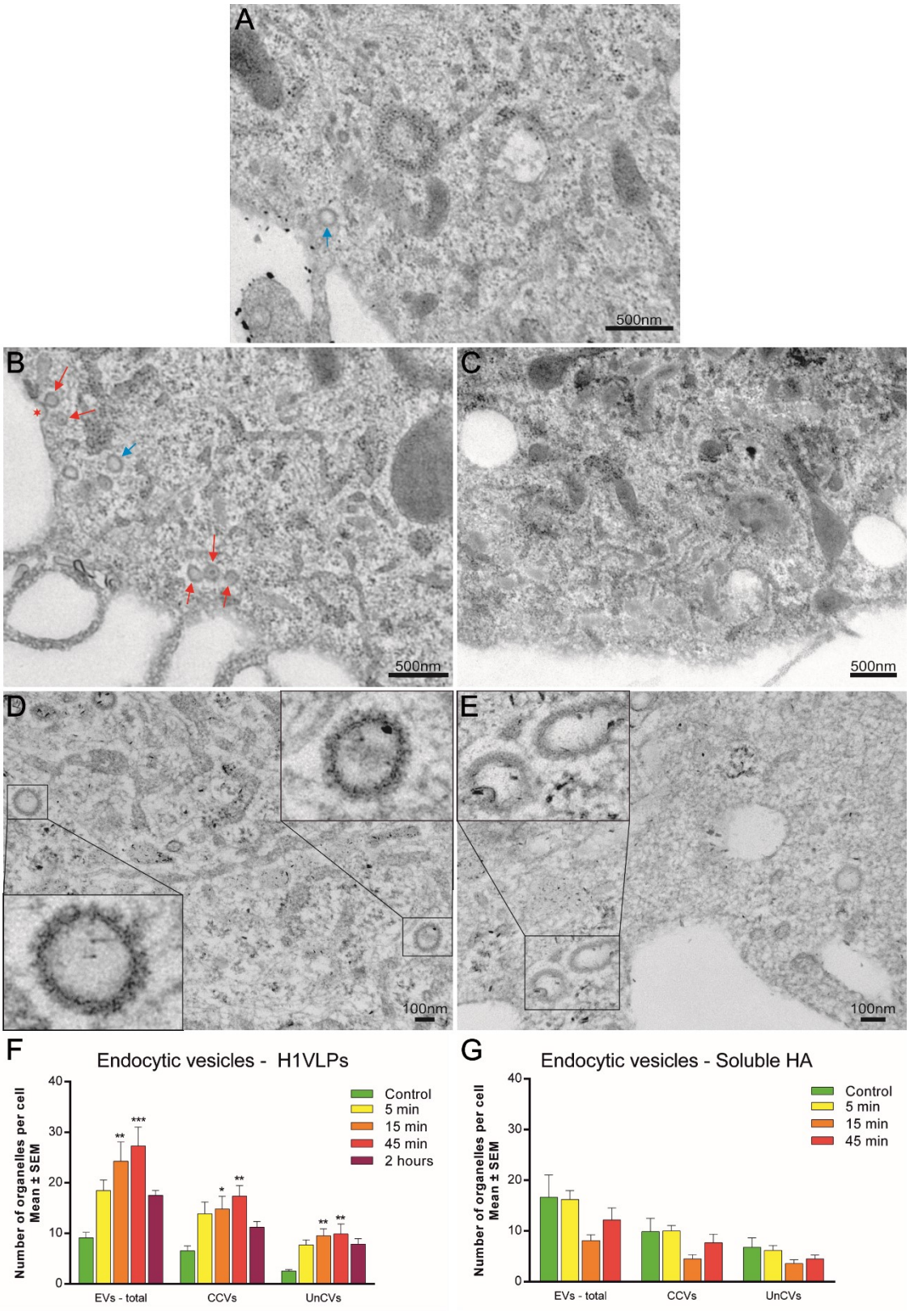

Figure 6. Quantification of endocytic vesicle formation stimulated by H1 VLPs or soluble HA protein in hMDMs. Legend overleaf.

Figure 6. Quantification of endocytic vesicle formation stimulated by H1 VLPs or soluble HA protein in hMDMs

Electron micrographs of hMDMs exposed to H1 VLPs or soluble HA protein are shown in A-E. Quantification of endocytic vesicles (EVs – total), clathrin-coated vesicles (CCVs), and uncoated vesicles (UnCVs) in 10 cells (hMDMs) exposed to either H1 VLPs or soluble HA are shown in E and F. (A) Control (non-exposed) hMDM showing the formation of only one uncoated vesicle (Blue arrow). (B) Endocytic vesicles formed in an hMDM exposed to H1 VLPs for 15 min. Red arrows show clathrin-coated vesicles; blue arrows show uncoated vesicles; red star indicates a forming endocytic vesicle. (C) hMDM exposed to soluble HA for 15 min. (D) Example of two clathrin-coated vesicles shown: these vesicles are characterized by a bristle-like (spiky) coat, corresponding to clathrin proteins. (E) Example of two uncoated vesicles shown: unlike clathrin-coated vesicles, uncoated vesicles do not display a spiked protein coat. (F) Quantification of endocytic vesicles formation in hMDMs exposed to H1 VLPs. (G) Quantification of endocytic vesicles formation in hMDMs exposed to soluble HA protein. *$p<0.05$; **$p<0.01$; ***$p<0.001$, compared to control – One-Way ANOVA with multiple comparisons. Data from two experiments were analyzed.

5.2. H1 VLPs may be internalized via clathrin-dependent endocytosis, caveolin-dependent endocytosis, and macropinocytosis, in hMDMs

We then assessed by confocal microscopy the endocytosis routes that the H1 VLPs utilize for internalization, using a series of drugs with inhibitory effects affecting different endocytic processes (detailed below), namely chlorpromazine, genistein, amiloride, and dynasore. The presence of H1 VLPs was detected at the surface of hMDMs using immuno-fluorescent labeling of HA. Because these immunofluorescence experiments were conducted on intact cells (in the absence of any permeabilizing agents), only those VLPs on (or outside) the cell surface were labeled and therefore detected. The changes in fluorescence were measured at different time points.

As a control, the internalization of hemagglutinin (HA) was followed over time in the absence of inhibitors (Figure 7 – Control; Figure 8A, 8B, 8C). The intensity of HA fluorescence was minimal at all the time points studied. Quantification showed a decrease from 5 min to 15 min, and then intensity remained low until 45 min (Figure 9). These observations suggest that H1 VLPs did not accumulate at the surface of hMDMs and were instead rapidly endocytosed; the fluorescence remained low (Figure 9) until later time points indicating that the VLPs are continuously internalized into hMDMs.

Chlorpromazine is a clathrin-mediated endocytosis (CME) specific inhibitor (Figure 7 – Chlorpromazine). At 5 min time point, chlorpromazine resulted in a significant increase in HA fluorescence compared to control (Figure 8A). This increase continued at 15 min (Figure 8B) and also was maximum at this time point, compared with the other time points (Figure 9 – Chlorpromazine). At 45 min the HA fluorescence had decreased to normal (Control) values (Figure 8C). These observations indicate that chlorpromazine is partially and initially inhibiting the internalization of H1 VLPs, demonstrating a partial role of CME in this process. The reduction

of HA fluorescence at the later time point (45 min) to normal values indicates that the H1 VLPs are eventually able to enter the hMDMs via alternative routes that do not involve CME.

Genistein is a caveolin-dependent endocytosis specific inhibitor (Figure 7 – Genistein). Genistein caused no significant effect at 5 min (Figure 8A) but a highly significant increase in HA fluorescence at 15 min (Figure 8B). At 15 min, the fluorescence was the highest (Figure 9 – Genistein) and it then went down to close to control values at 45 min (Figure 8C). This pattern was similar to that seen above in the presence of chlorpromazine, suggesting a partial role of caveolin-dependent endocytosis in the internalization of the VLPs; likewise, the later reduction in fluorescence indicates that these particles are also able to enter the cell through processes independent of caveolin.

Amiloride is a macropinocytosis inhibitor (Figure 7 – Amiloride). Similarly to genistein, amiloride's effect was not noticeable at 5 min (Figure 8

A). However, as seen with the previous drugs, amiloride provoked an increase in HA fluorescence at 15 min (Figure 8B), and then went down at 45 min (Figure 8C). As for chlorpromazine and genistein, amiloride's strongest effect was at 15 min, when HA fluorescence was maximum compared to other time points (Figure 9 – Amiloride). This suggests that amiloride is also partially disrupting the internalization of H1 VLPs, although they are eventually taken up by hMDMs, demonstrating a partial role of macropinocytosis in this mechanism.

Dynasore was the last inhibitor used. Unlike all the other drugs shown so far, dynasore inhibits, at the same time, clathrin-dependent endocytosis (CME), clathrin-independent endocytosis (CIE) (which includes caveolin-dependent endocytosis), and macropinocytosis together. This generalized inhibition of endocytosis mechanisms affected H1 VLPs' internalization in a

characteristic manner, visibly distinguishable from other conditions: at all the time points shown, dynasore induced the formation of extracellular material where HA fluorescence accumulated (Figure 7 – Dynasore). The accumulation of HA-labeling on the cell membrane and the formation of HA fluorescence-rich extracellular material were further analyzed by performing immuno-gold labeling of HA of the same samples, and electron micrographs were collected (Figure 10), confirming the observations. Additionally, dynasore induced an increment in HA fluorescence that was comparatively higher than the control conditions at all the time points studied (Figure 8A, 8B, 8C). Not only the fluorescence was higher at all the time points, but it had raised from 5 min to 15 min and had raised even more at 45 min (Figure 9 – Dynasore). Dynasore-induced inhibition was gradual, increasing over time, and it did not decrease at any of the time points evaluated, which indicate that blocking CME, CIE, and macropinocytosis altogether had a major and continuous effect in the internalization of H1 VLPs. The characteristic extracellular material rich in HA fluorescence generated by the addition of dynasore are also coincident with a major effect in the uptake of VLPs into hMDMs.

In summary, inhibition of CME, caveolin-dependent endocytosis, and macropinocytosis by chlorpromazine, genistein, and amiloride (respectively) had a partial effect in the internalization of H1 VLPs, characterized by an initial accumulation of HA fluorescence on the hMDMs surface, reaching its highest at 15 min, and a subsequent decrease in such accumulation between 15 min and 45 min. On the other hand, when all CME, CIE, and macropinocytosis were inhibited by the addition of dynasore, the HA fluorescence increased gradually over time, reaching the highest value at 45 min. These data together with the generation of extracellular material with accumulations of HA induced by dynasore, suggest a major inhibitory effect caused by this drug. Altogether, the results presented in this section indicate that the H1 VLPs may utilize diverse

mechanisms to enter hMDMs, including CME, caveolin-dependent endocytosis, and macropinocytosis.

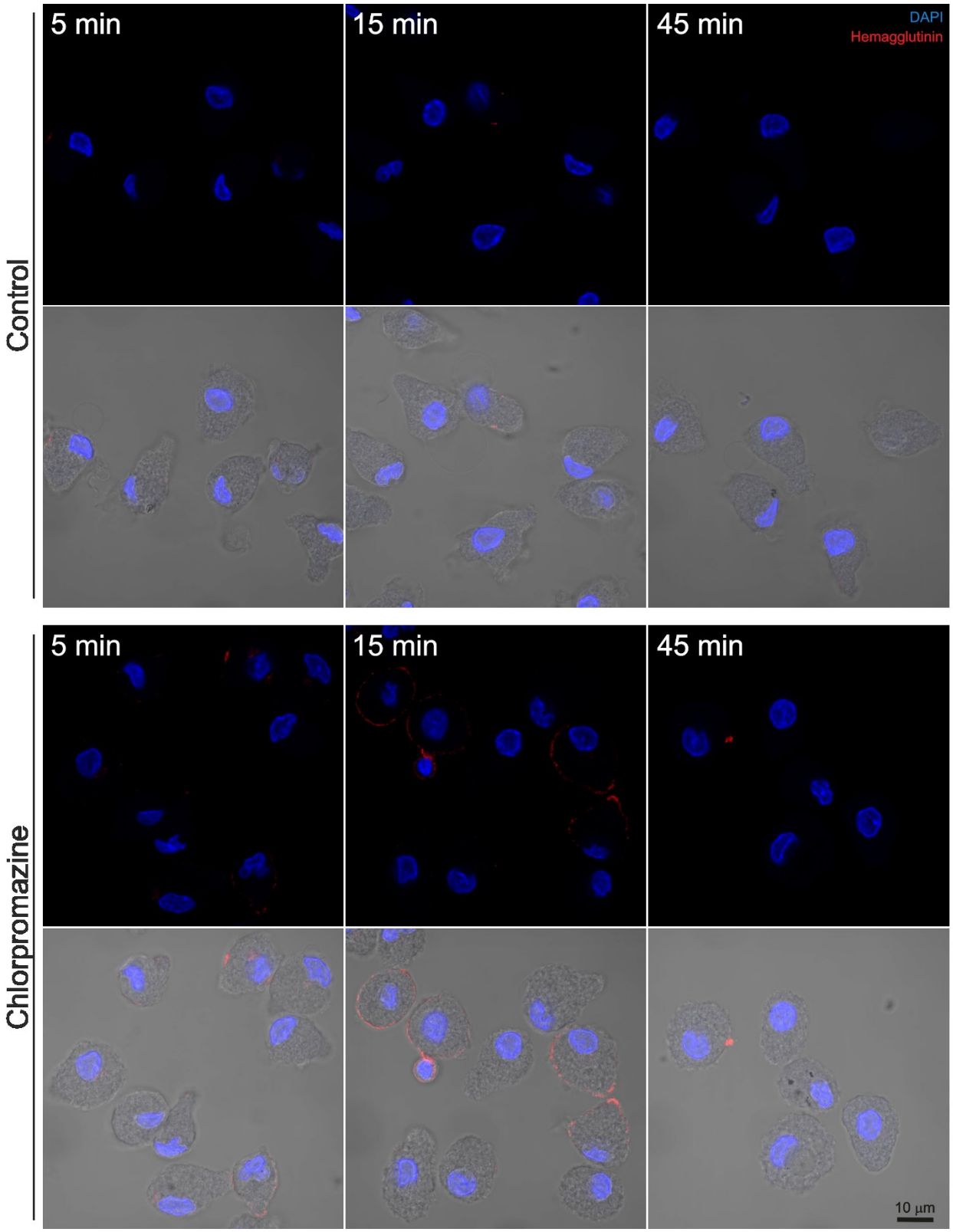

Figure 7. Effects of endocytosis inhibitors in the internalization of H1 VLPs in hMDMs. Legend overleaf.

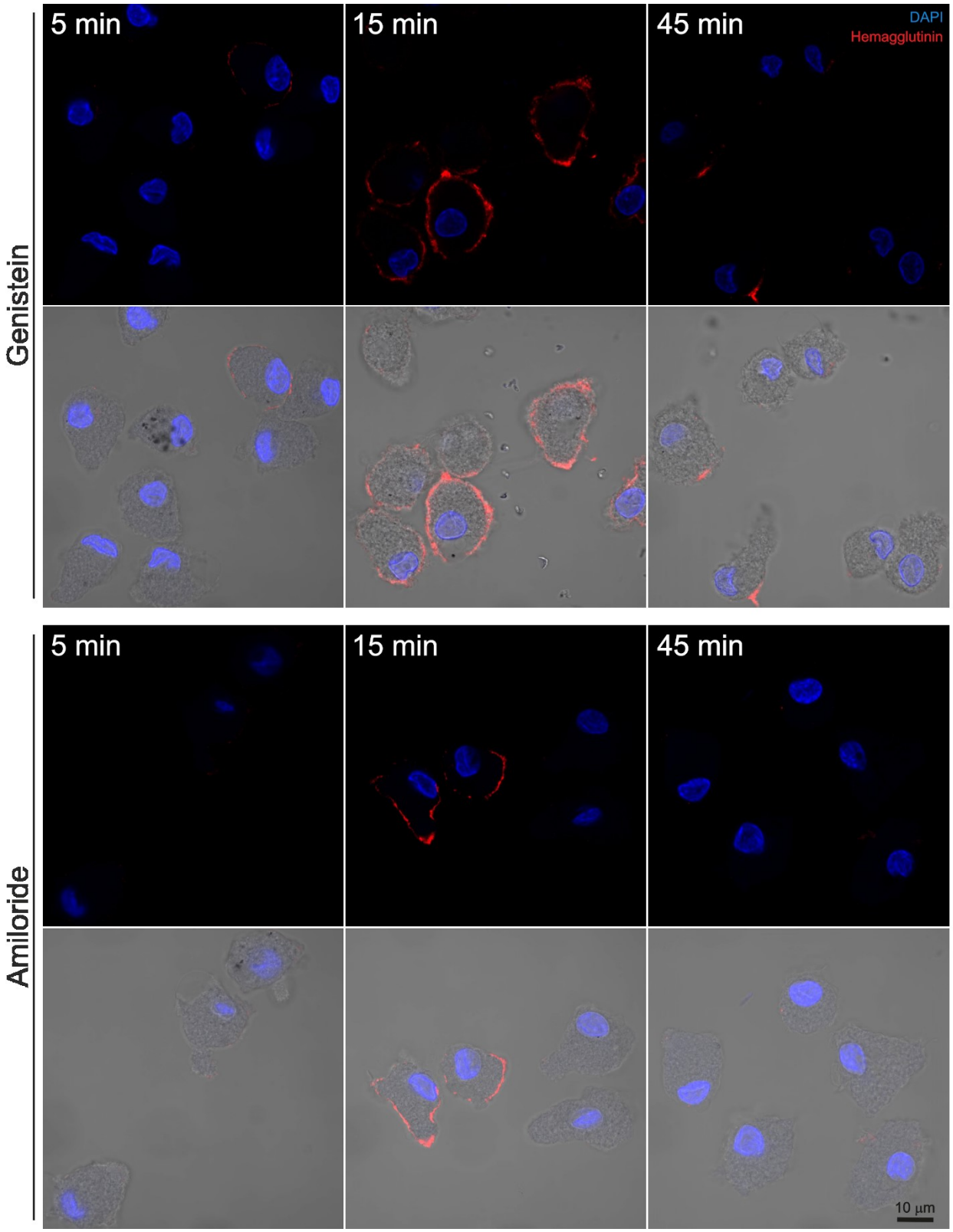

Figure 7. Effects of endocytosis inhibitors in the internalization of H1 VLPs in hMDMs. Legend overleaf.

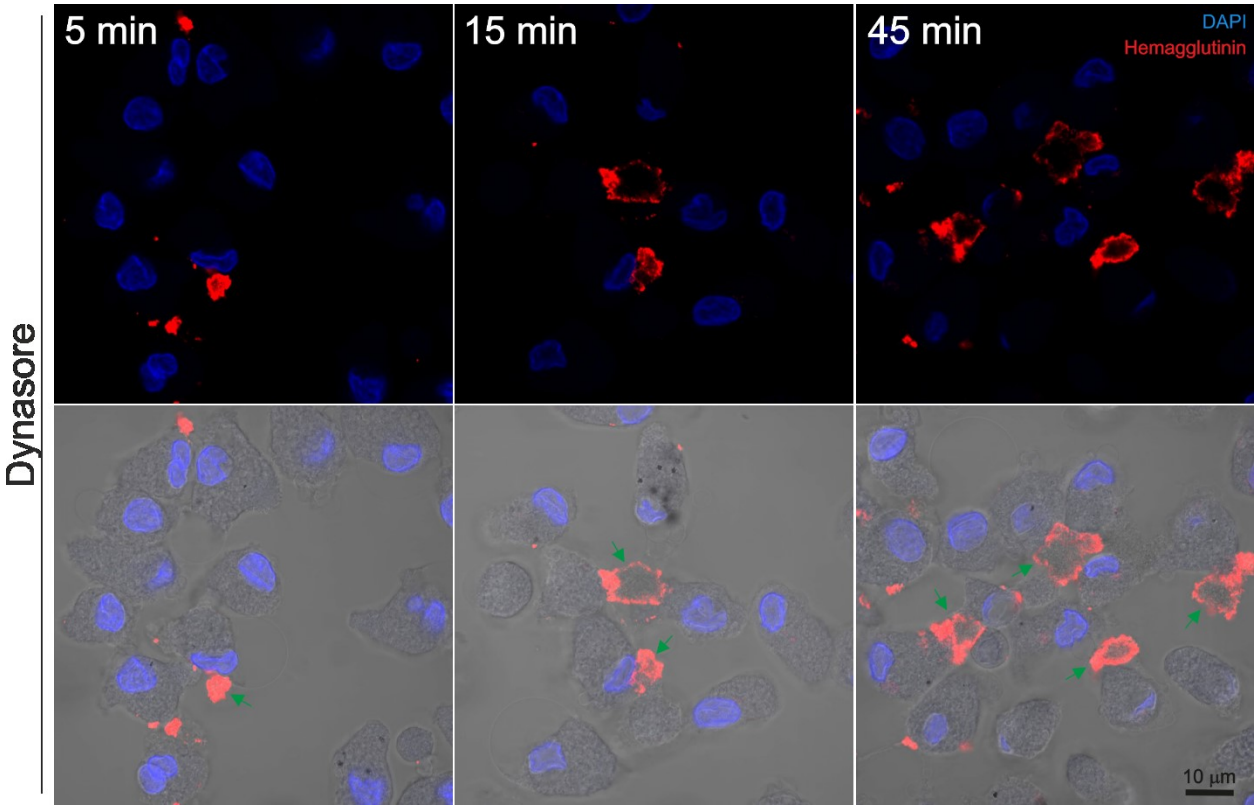

Figure 7. Effects of endocytosis inhibitors in the internalization of H1 VLPs in hMDMs

Confocal microscopy images of hMDMs incubated with H1 VLPs for 5 min, 15 min, or 45 min, and in the presence of chlorpromazine, genistein, or amiloride, dynasore, or in the absence of inhibitors (Control). Top panels show Red and Blue fluorescence channels, for Hemagglutinin and DAPI, respectively; Bottom panels have the transmitted light channel superimposed additionally. Note that dynasore inhibition induces the formation of hemagglutinin-rich extracellular material (Green arrows).

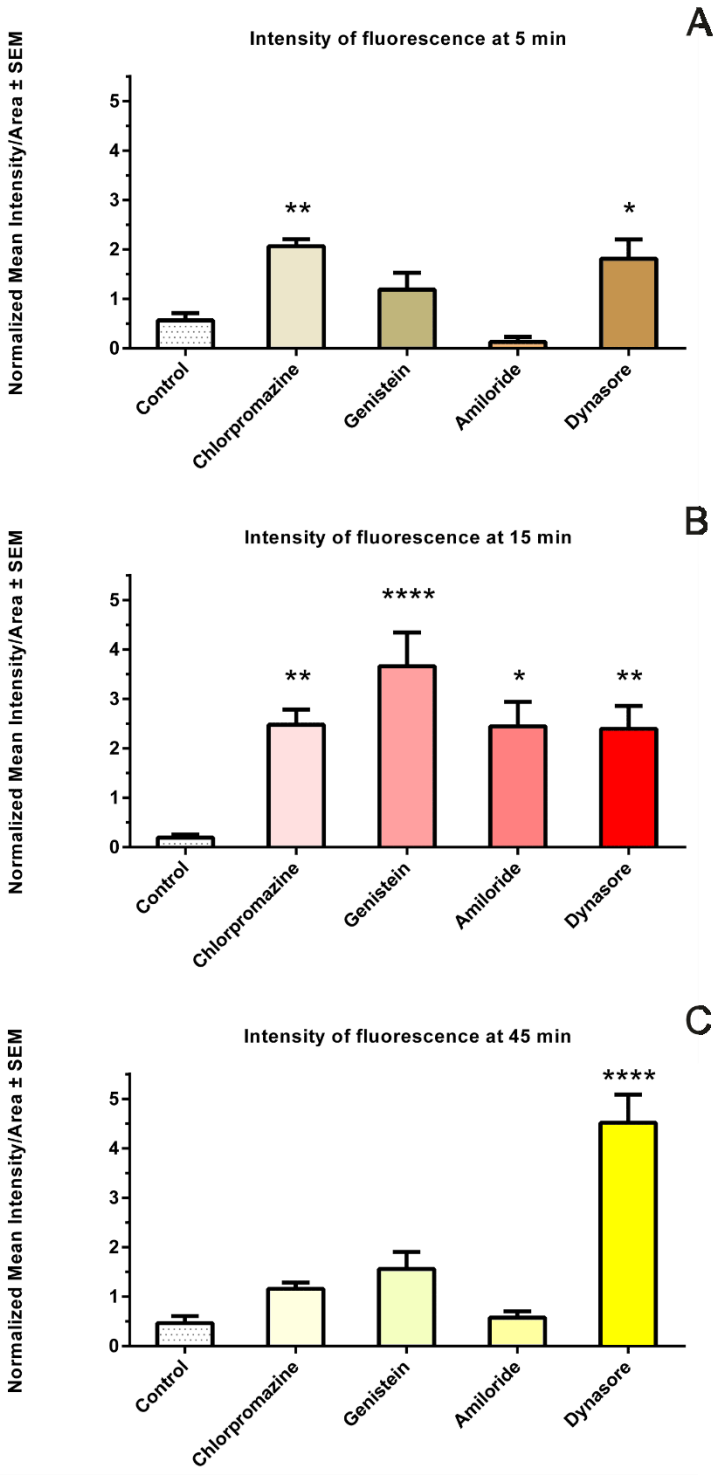

Figure 8. Quantification of the effects of endocytosis inhibitors in the internalization of H1 VLPs in hMDMs. Legend overleaf.

Figure 8. Quantification of the effects of endocytosis inhibitors in the internalization of H1 VLPs in hMDMs

The inhibitory effect of different endocytosis inhibitors in the internalization of H1 VLPs was indirectly assessed by the accumulation of HA fluorescence at the cell surface of hMDMs in the presence of the different drugs. This effect was evaluated by measuring the mean intensity of HA fluorescence per cell area on confocal microscopy images of hMDMs incubated with H1 VLPs in the presence of chlorpromazine, genistein, or amiloride, dynasore, or in the absence of inhibitors (Control). The values were measured in an average of 56 cells per condition and per time point. (A) Quantification of HA fluorescence in hMDMs incubated with H1 VLPs for 5 minutes. (B) Quantification of HA fluorescence in hMDMs incubated with H1 VLPs for 15 minutes. (C) Quantification of HA fluorescence in hMDMs incubated with H1 VLPs for 45 minutes. *$p<0.05$; **$p<0.01$; ****$p<0.001$, compared to the control – One-Way ANOVA with multiple comparisons. Data from three experiments were analyzed.

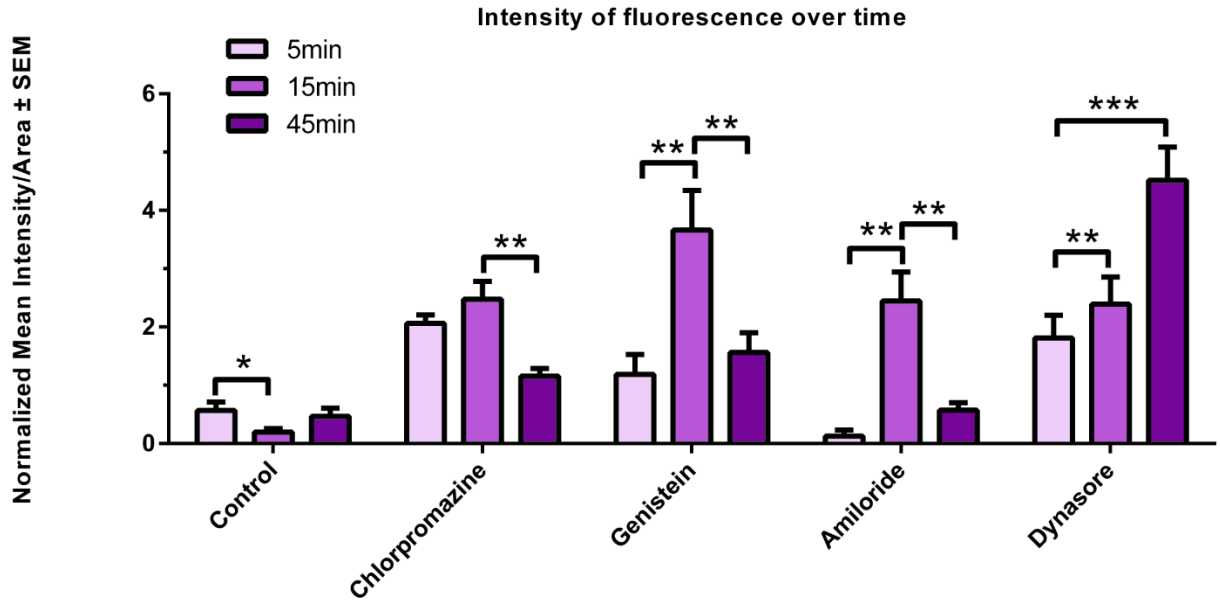

Figure 9. Quantification of the effects of endocytosis inhibitors over time in the internalization of H1 VLPs in hMDMs.

Alternative plot of the data presented on Figure 9. The changes in intensity of HA fluorescence values over time are represented, for hMDMs incubated with H1 VLPs for 5 min, 15 min, and 45 min, in the presence of chlorpromazine, genistein, or amiloride, dynasore, or in the absence of any drugs (Control). Values of intensity of fluorescence for each inhibitor are compared between the different time points. *$p<0.05$; **$p<0.01$; ***$p<0.001$ – One-Way ANOVA with multiple comparisons. Data from three experiments were analyzed.

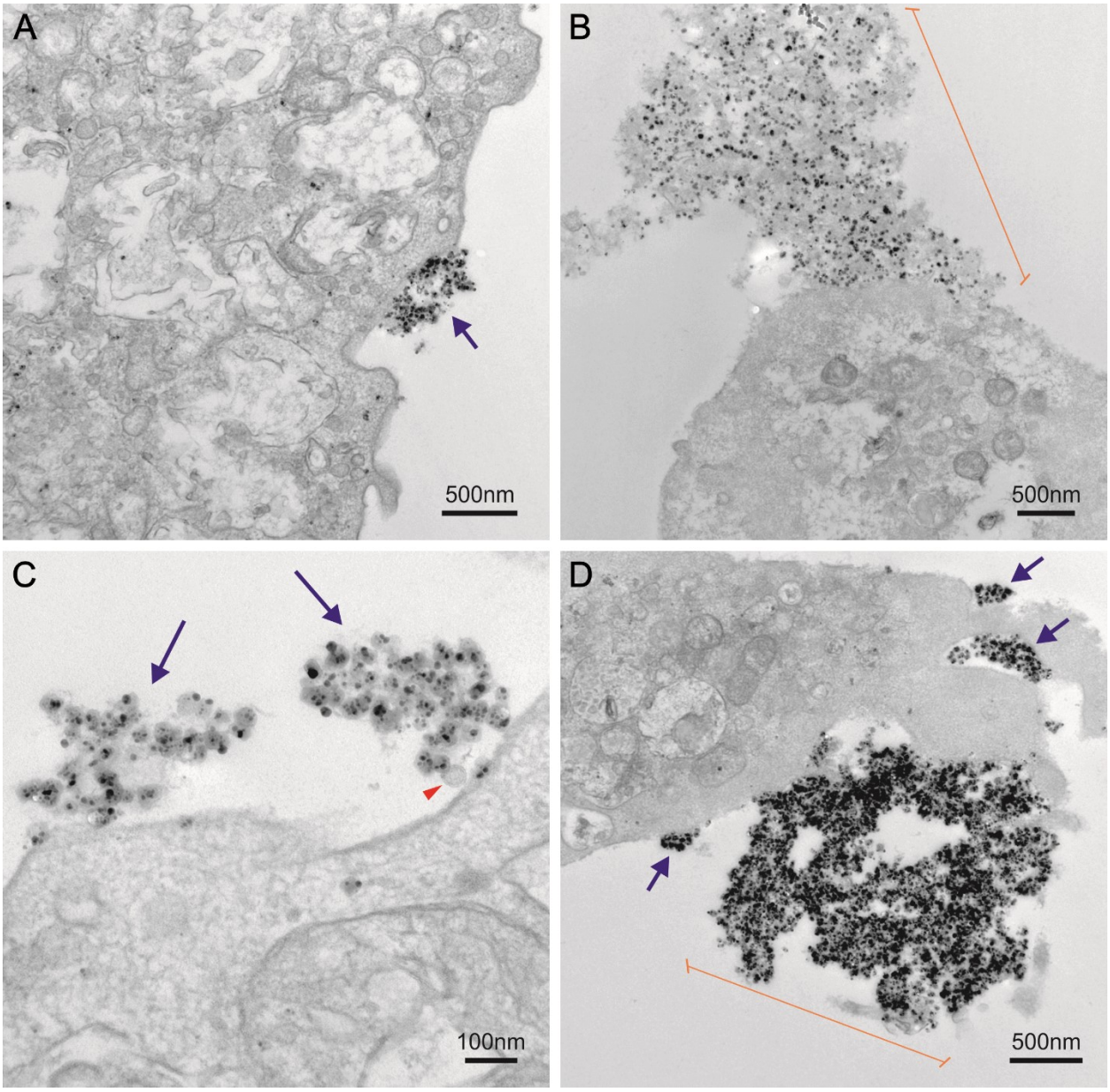

Figure 10. Effects of dynasore in the internalization of H1 VLPs in hMDMs

Immuno-gold labelling and silver enhancement performed on the samples shown in Figure 7. Here are electron micrographs of hMDMs incubated with H1 VLPs for 5 min in the presence of dynasore, showing two features: smaller accumulations of hemagglutinin at the cell surface (purple arrows) and much bigger accumulations of hemagglutinin in extracellular material (delimited in orange). Note a discus-shaped double-membraned structure on (C) (Red arrowhead), possibly an H1 VLP.

5.3. H1 VLPs stimulate endosomal organelle formation in hMDMs

The observation that H1 VLPs were internalized along various pathways suggests that these particles may also be processed for antigen presentation in diverse manners. Here, we observed if the VLPs were able to stimulate the formation of different organelles involved in endosomal trafficking, using electron microscopy.

Early endosomes (EEs) look like empty vesicles that generally span between 500nm and 1μm in diameter (Figure 11A and 11B). A subgroup of early endosomes can be distinguished by electron microscopy: these are clathrin-coated early endosomes (CCEEs), characterized by the presence of a dark area in their membrane, corresponding to a clathrin lattice (Raposo *et al.*, 2001) (Figure 11B). When cargo is to be directed towards the lysosomal pathway for degradation, the early endosome matures and becomes a late endosome that may take the form of multi-vesicular body (MVBs), formed by the invagination of the endosomal membrane and pinching inwards (Piper and Katzmann, 2007): MVBs can be visualized on electron micrographs as vesicles that contain other vesicles inside, or sometimes protein debris (Figure 11A and 11C). When extracellular material is taken up by pinocytosis, the resulting vesicles may fuse with the lysosome and become heterolysosomes (HLs) (Mego and McQueen, 1967): we considered heterolysosomes those vesicles that contained lysosomes inside – lysosomes have been defined in electron microscopy by their dark appearance (electron dense organelles) (Figure 11A and 11D). These four organelles were quantified in hMDMs incubated with H1 VLPs (Figure 11E) or soluble HA (Figure 11G) for 5 min, 15 min, 45 min, or 2 hours.

The number of early endosomes increased significantly after 15 min of incubation of hMDMs with H1 VLPs as compared to the control cells (not incubated with H1 VLPs). The count decreased at later time points (45 min) to control values (Figure 11E). However, when the cells were incubated

with soluble HA protein, the count of early endosomes did not vary significantly at any incubation time points (Figure 11F).

Similarly, clathrin-coated early endosomes significantly went up in hMDMs incubated with H1 VLPs for 15 min (Figure 11E) and down at later time points, whereas incubation with soluble HA didn't result in any significant change in the number of CCEEs at the time points studied (Figure 11F).

The number of multi-vesicular bodies did not increase or decrease significantly at any incubation time points, in comparison with the control cells group, either after incubation with H1 VLPs (Figure 11E) or with soluble HA (Figure 11F).

When heterolysosomes were quantified, no significant variation in their number was observed at any of the time points analyzed, neither when the hMDMs were incubated with H1 VLPs (Figure 11E) nor when they were incubated with soluble HA protein (Figure 11F).

In summary, incubation of hMDMs with H1 VLPs was able to stimulate the formation of early endosomes and clathrin-coated early endosome significantly soon after incubation (15 min). The formation of multi-vesicular bodies or heterolysosomes were not stimulated by the incubation with H1 VLPs. Soluble HA protein did not alter significantly the number of any of the organelles, at any of the incubation time points studied.

These data suggest that the H1 VLPs are delivered to early endosomes and clathrin-coated early endosomes soon after internalization. This is consistent with the results shown in the previous sections which suggest that H1 VLPs are endocytosed into hMDMs through multiple pathways.

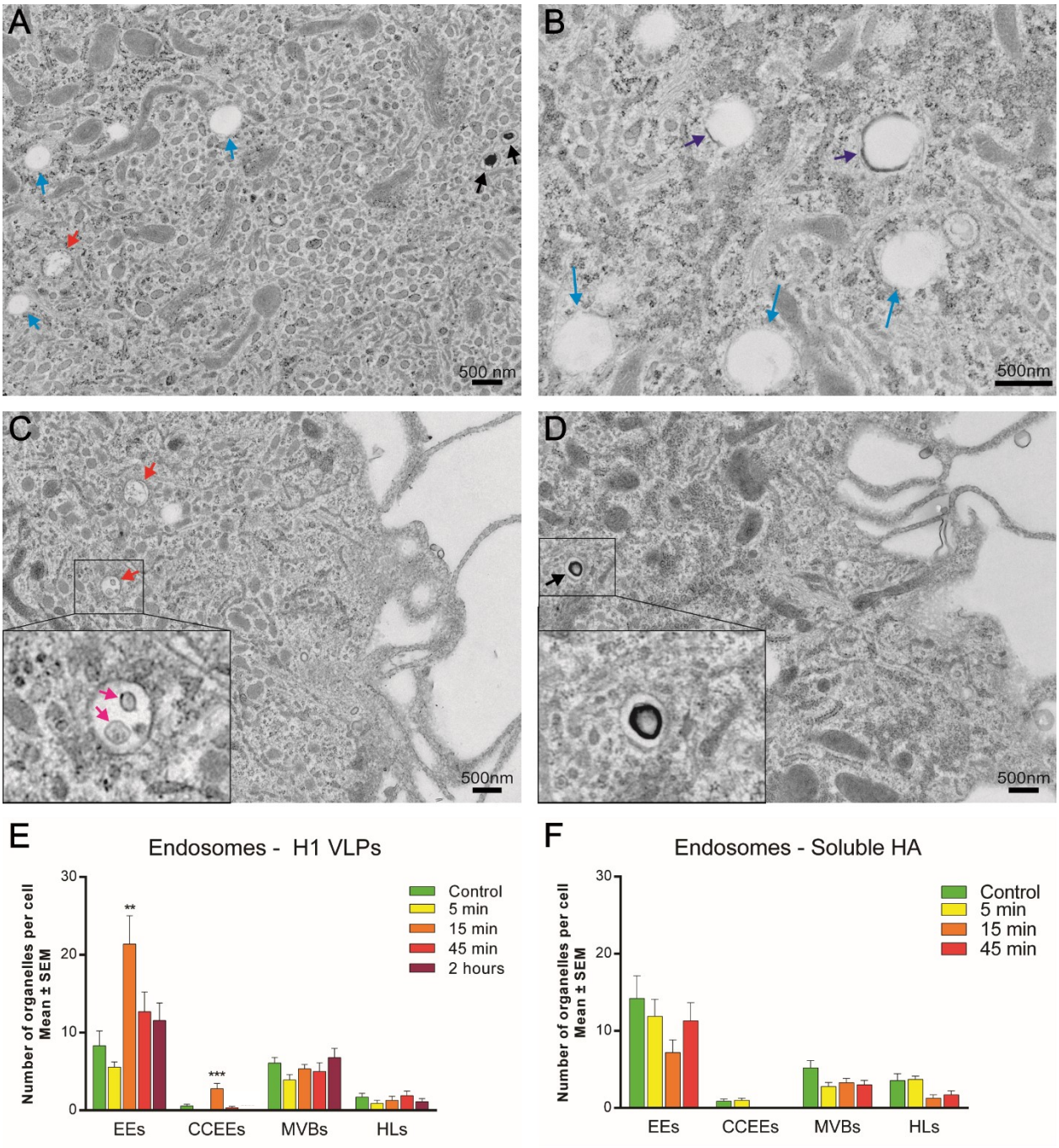

Figure 11. Quantification of endosomal organelle formation stimulated by H1 VLPs or soluble HA protein in hMDMs. Legend overleaf.

Figure 11. Quantification of endosomal organelle formation stimulated by H1 VLPs or soluble HA protein in hMDMs

Electron micrographs of hMDMs incubated with H1 VLPs (A-D). (A) Electron micrograph showing different organelles: early endosomes, heterolysosomes, and multi-vesicular bodies are indicated in blue, red, and black arrows, respectively. (B) Electron micrograph showing early endosomes (light blue arrows) and clathrin-coated early endosomes (purple arrows). (C) Electron micrograph showing multi-vesicular bodies (red arrows). Magenta arrows indicate vesicles inside the multi-vesicular body. (D) Electron micrograph showing one example of a heterolysosome, indicated by a black arrow. (E) and (F) show the quantification of endosomal organelles present in hMDMs exposed to H1 VLPs and soluble HA, respectively. **$p<0.01$; ***$p<0.001$, compared to control – One-Way ANOVA with multiple comparisons. Data from two experiments were analyzed.

5.4. Hemagglutinin in the form of H1 VLPs may be delivered to different endosomal compartments involved in antigen presentation

The electron microscopy analyses showed that incubation with H1 VLPs resulted in upregulation of the formation of early endosomes and clathrin-coated early endosomes. Here, we sought to further study the intracellular fate of the particles by tracking their HA using immuno-labeling.

Soluble HA was also used as a comparator in the experiments presented in this section, however, the fluorescence of HA delivered as soluble HA had almost completely disappeared at 45 min in all the conditions analyzed, so the comparison between earlier and later time points could not be made (data not shown).

First, we analyzed the co-localization of HA and early endosome antigen 1 (EEA1), an early endosomal marker (Selby *et al.*, 2017) (Figure 12A) and the colocalization of HA and clathrin (Figure 12B) in hMDMs that had been exposed to H1 VLPs for 15 min and for 45 min. This confirmed that H1 VLPs were delivered to early endosomes and clathrin-coated early endosomes: HA and EEA1 fluorescence showed great co-localization that was very significantly higher at 15 min than at 45 min (Figure 12C). HA and clathrin fluorescence co-localization was also significantly higher at 15 min and decreased at 45 min (Figure 12C). However, the co-localization coefficient of HA and clathrin was lower than that of HA and EEA1. This is consistent with the clathrin coat being present in a fraction of the early endosomes.

These co-localization analyses indicate that H1 VLPs are being initially delivered to early endosomes, part of which are clathrin-coated early endosomes. After some time, the HA present in these early endosomes decreases, indicating that it is being mobilized to other intracellular compartments or partially degraded.

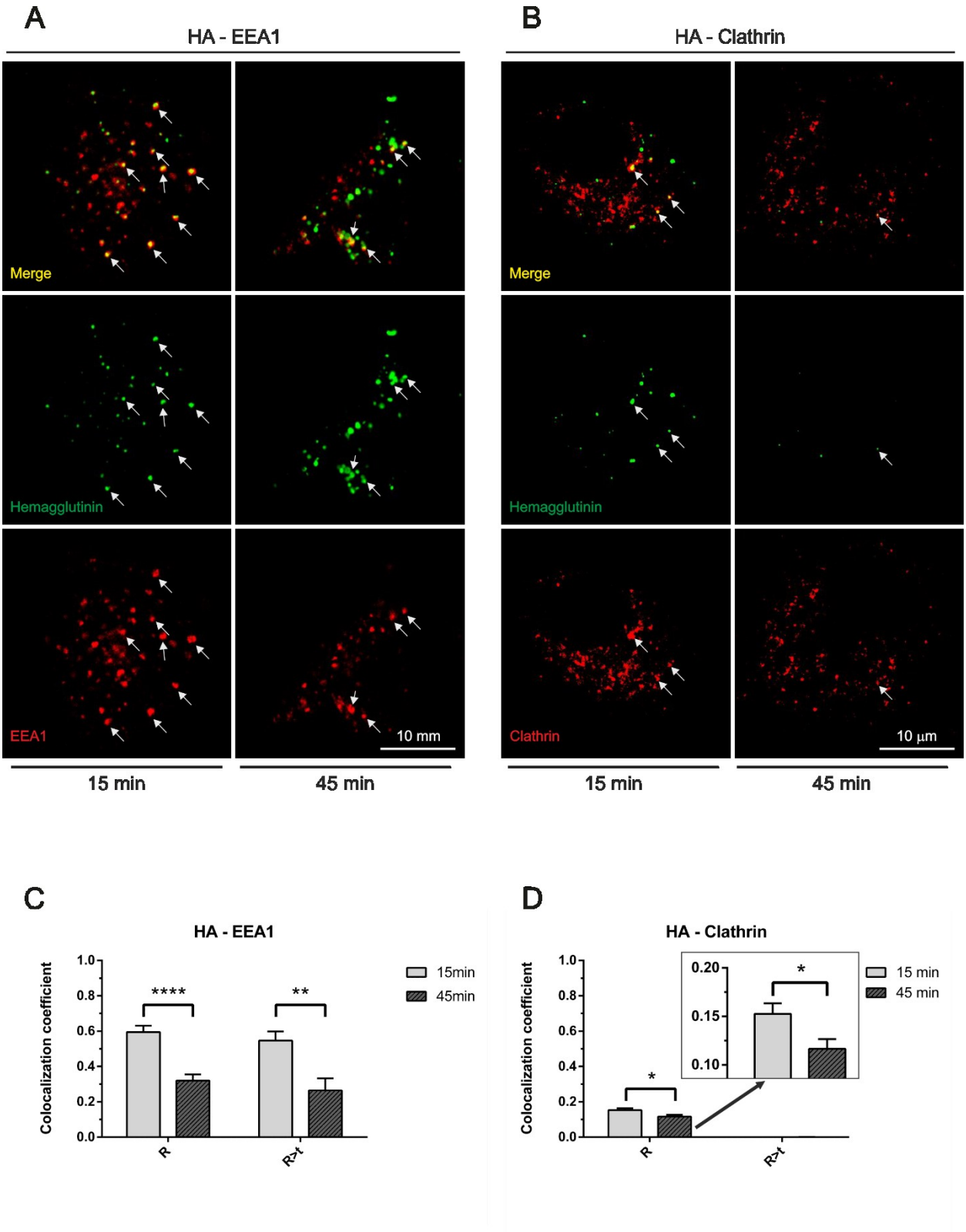

Figure 12. Colocalization analyses of HA with early endosomal markers. Legend overleaf.

Figure 12. Colocalization analyses of HA with early endosomal markers

(A) Confocal microscopy images of hMDMs incubated with H1 VLPs for 15 min (left column) and 45 min (right column). HA is shown in green; EEA1 is shown in red. (B) Confocal microscopy images of hMDMs incubated with H1 VLPs for 15 min (left column) and 45 min (right column). HA is shown in green; clathrin is shown in red. (C) and (D) are the co-localization coefficient values calculated from (A) and (B), respectively. R indicates Pearson's correlation coefficient; R>t indicates Pearson's correlation coefficient above threshold. *$p<0.05$; **$p<0.01$; ****$p<0.0001$ – Unpaired T-test. Data from three experiments were analyzed.

The first possible destiny for any cargo delivered to the early endosome is to follow the endosome maturation pathway. This process leads to fusion of the early endosome with the lysosome, which causes acidification and eventual degradation of the cargo. We used co-localization analyses to assess whether the HA from the H1 VLPs followed this pathway and was directed towards the lysosome for degradation.

HA and LAMP1 (a lysosomal marker) (Eskelinen, 2006) co-localized moderately at 15 min and this co-localization significantly increased at 45 min (Figure 13A, 13B). The increase of co-localization of fluorescence of HA and LAMP1 from early to late time points is consistent with the decrease in co-localization between HA and EEA1 over time. Taken together, these results indicate that the lysosomes may be receiving HA from early endosomes. Nevertheless, the total co-localization of fluorescence between HA and LAMP1 is lower than between HA and EEA1, which suggests that HA is also being delivered to compartments other than lysosomes or partially degraded.

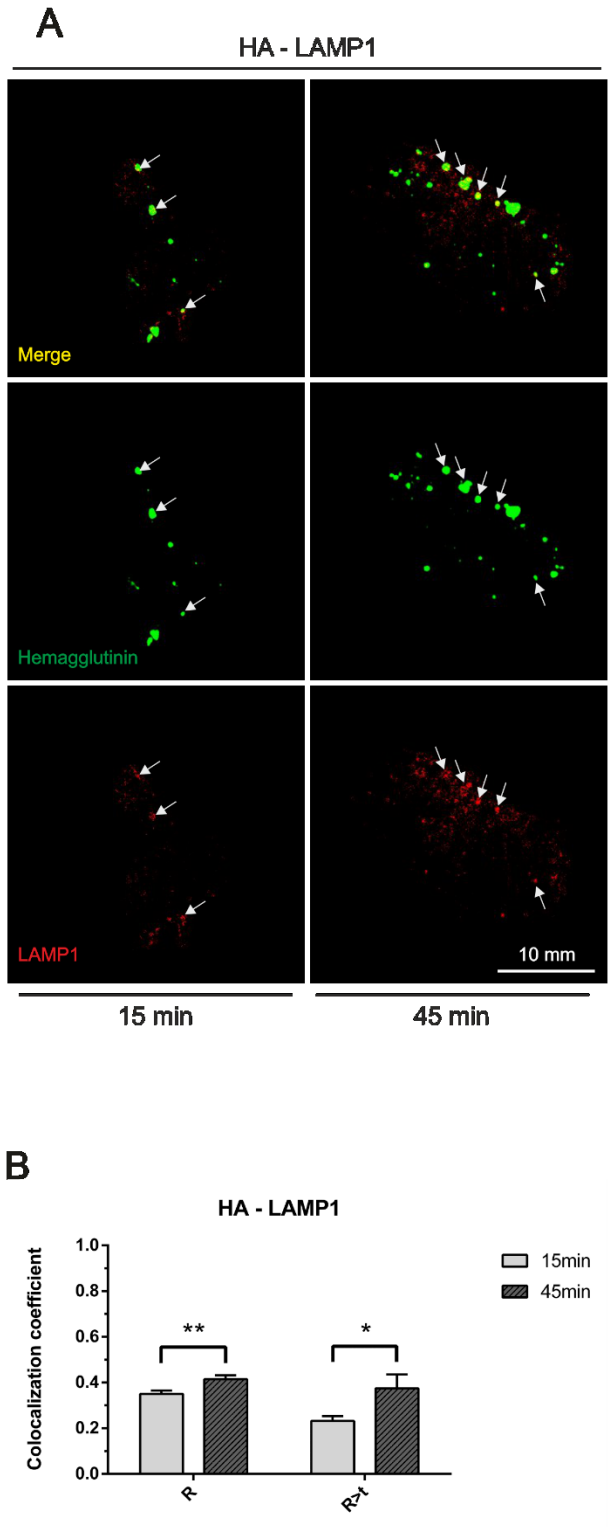

Figure 13. Colocalization analyses of HA with a lysosomal marker. Legend overleaf.

Figure 13. Colocalization analyses of HA with a lysosomal marker

(A) Confocal microscopy images of hMDMs incubated with H1 VLPs for 15 min (left column) and 45 min (right column). HA is shown in green; EEA1 is shown in red. (B) is the quantification of the co-localization coefficient values calculated from (A). R indicates Pearson's correlation coefficient; R>t indicates Pearson's correlation coefficient above threshold. *$p<0.05$; **$p<0.01$ – Unpaired T-test. Data from three experiments were analyzed.

The next possibility for particles delivered to the early endosome is to be recycled back to the membrane, through recycling endosomes. The existence of two different recycling pathways has been discussed. On one hand, there is a slow recycling pathway: the recycling compartments involved are characterized by the presence of different Rab proteins, such as Rab3c, and have been shown to be involved in antigen cross-presentation (Zou *et al.*, 2009). On the other hand, there is a rapid recycling compartment, which involves Rab4-positive vesicles that recycle material back to the membrane, sometimes even before reaching the early endosome (Maxfield and McGraw, 2004). This pathway is not generally associated with MHC I cross-presentation.

The co-localization of HA with Rab3c was very significantly higher at 15 min than at 45 min (Figure 14C). The co-localization between HA and Rab4 was moderate at both time points studied and did not decrease significantly over time (Figure 14D).

On one side, the initial higher co-localization of HA and Rab3c suggests that part of the HA delivered as H1 VLPs is initially directed to Rab3c-positive recycling endosomes, and the decrease of co-localization indicates that the HA delivered into these compartments is degraded or directed back to the membrane before 45 min are reached. On the other side, part of HA appears in Rab4-positive endosomes that quickly recycle cargo back to the plasma membrane.

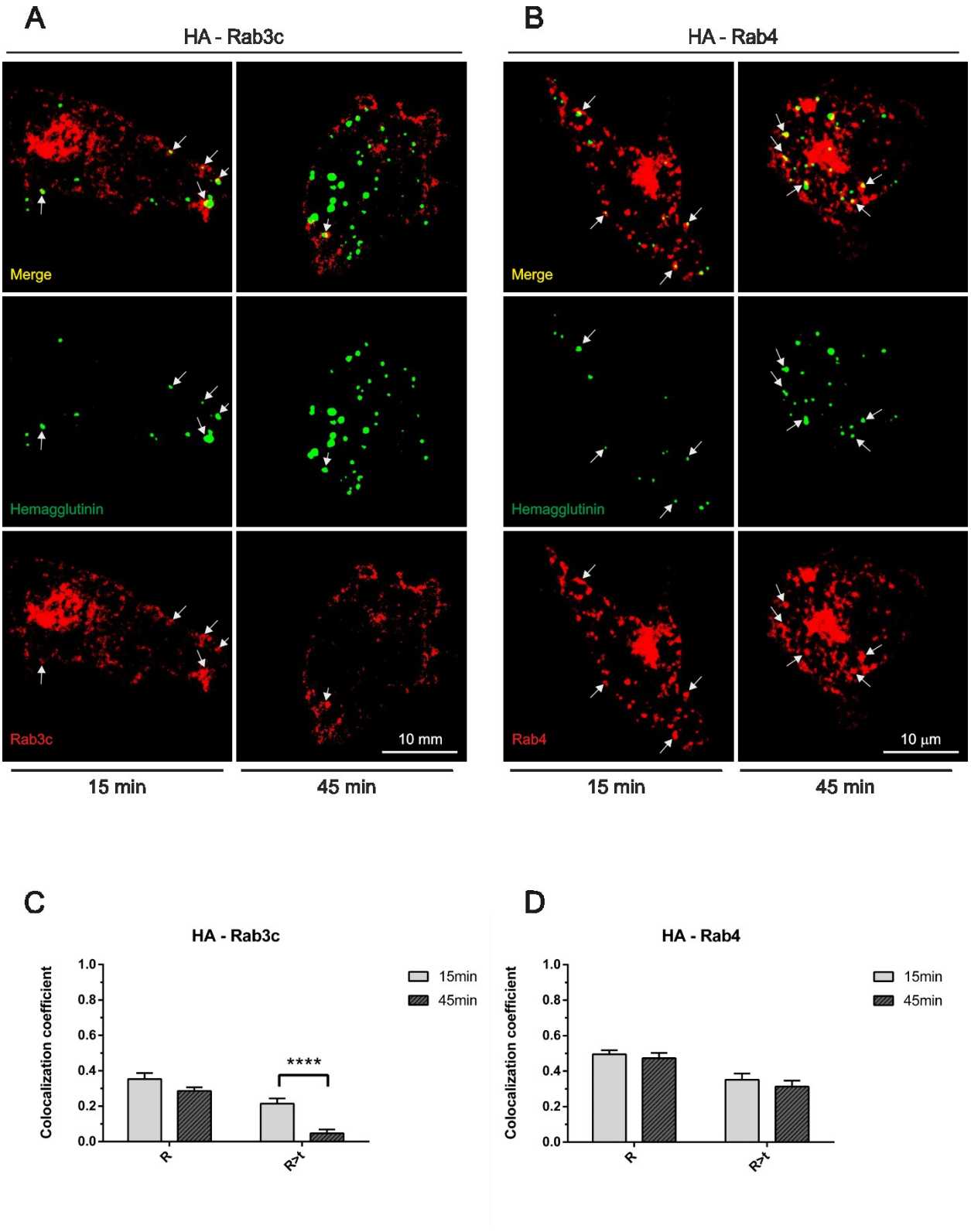

Figure 14. Colocalization analyses of HA with recycling endosomal markers. Legend overleaf.

Figure 14. Colocalization analyses of HA with recycling endosomal markers

(A) Confocal microscopy images of hMDMs incubated with H1 VLPs for 15 min (left column) and 45 min (right column). HA is shown in green; Rab3c is shown in red. (B) Confocal microscopy images of hMDMs incubated with H1 VLPs for 15 min (left column) and 45 min (right column). HA is shown in green; Rab4 is shown in red. (C) and (D) are the co-localization coefficient values calculated from (A) and (B), respectively. R indicates Pearson's correlation coefficient; R>t indicates Pearson's correlation coefficient above threshold. ****$p<0.0001$ – Unpaired T-test. Data from three experiments were analyzed.

In summary, the results presented in this section suggest that the H1 VLPs are internalized and delivered mostly to the early endosomal compartment, in hMDMs. Part of the HA delivered in this manner is recycled back to the membrane in Rab4-positive endosomes. More interestingly, part of the early endosomes into which H1 VLPs are taken up in hMDMs are clathrin-coated; these clathrin-coated regions on early endosomes have been related to protein sorting towards the lysosome. Consistent with this idea, part of the HA delivered to the early endosome is being directed towards the lysosome. On the other hand, part of the HA internalized into these cells may be also moved into Rab3c-positive recycling endosomes.

Taken together, all these results suggest that HA delivered in the form of H1 VLPs can be degraded in multiple manners: in the lysosome, following the "classical" degradation pathway; and in Rab3c-positive recycling endosomes, following the alternative "cross-presentation" pathway.

6. DISCUSSION, CONCLUSION, AND FUTURE DIRECTIONS

6.1. Summary and discussion

Influenza infections cause many deaths and cases of severe illness worldwide, every year, which presents an obvious social, economic, and health burden (Peasah *et al.*, 2013). Vaccination is the best means to prevent influenza, but the different available vaccines vary in efficacy every season as new viral strains appear among the population (Ortiz *et al.*, 2013). Groups at risk like the elderly population also respond to vaccines differently. These problems prompt research in the direction of seeking better vaccines (Scorza *et al.*, 2016).

Virus-like particles bearing influenza hemagglutinin obtained by the expression of this protein within plant cells resemble the wild-type virus (D'Aoust *et al.*, 2010), while lacking any replicative capacity due to the fact that they do not contain viral RNA. Recent studies using cryo-electron microscopy have shown how the morphology of these VLPs is in fact similar to that of the native influenza virus and that the hemagglutinin molecules they bear are accessible to the immune system (Lindsay *et al.*, 2018). It has been shown as well that these particles recapitulate viral interactions with immune cells: the VLPs bind to the cell surface in a sialic acid-dependent manner, and they are then internalized in an energy-dependent fashion (Makarkov *et al.*, 2017).

More importantly, hemagglutinin-bearing VLPs are able to elicit strong and cross-reactive humoral and cell-mediated immune responses in both animal (D'Aoust *et al.*, 2008; Hodgins *et al.*, 2017) and humans as demonstrated in different clinical studies (Landry *et al.*, 2014; Pillet *et al.*, 2015; Pillet *et al.*, 2016). Besides, influenza VLPs remain stable up to 12 months when kept at

4°C (Lindsay *et al.*, 2018). All these characteristics in addition to the short time of production make these particles a promising candidate as a vaccine.

Despite the number of studies already conducted on these particles, the potent and diverse yet unusual immunogenicity generated by influenza hemagglutinin-bearing VLPs is still not completely understood.

Upon administration, one of the first cell types that make contact with a vaccine are antigen-presenting cells, as would be the case after exposure to the wild-type virus. Although they are part of the innate immune system, antigen-presenting cells are responsible for triggering the adaptive immune responses: more specifically, cell types such as dendritic cells and macrophages are often called "professional" antigen-presenting cells, as they can take up and process antigens, load them on major histocompatibility complex (MHC) molecules, and present them on their cell membrane to lymphocyte T cells (Unanue, 1984; Thery and Amigorena, 2001). This leads to the T lymphocytes' activation, which will help coordinate the effector immune response and immune memory (Kaech and Cui, 2012; Huang *et al.*, 2013). The generation of immune memory is crucial for vaccination, and memory T cells together with memory B cells are the main orchestrators of this function. Because T lymphocytes can only gain contact with epitopes presented on MHC molecules (Bjorkman *et al.*, 1987), the role of antigen-presenting cells is of very high importance during vaccination.

Hemagglutinin-bearing VLPs have been shown to elicit a response from both CD4+ and CD8+ T lymphocyte subtypes (Landry *et al.*, 2014), however, how the immune system handles hemagglutinin for presentation to these lymphocytic cell subtypes remains unclear. To better understand this process, we sought to determine how human macrophages internalize hemagglutinin-bearing VLPs and, after internalization, how the intracellular pathways followed

by hemagglutinin might influence its presentation on MHC I or MHC II molecules that ultimately lead to activation of CD8+ T cells or CD4+ T cells, respectively.

For our experiments, we used hemagglutinin 1 (H1) virus-like particles (VLPs) as a model for influenza vaccine, manufactured by Medicago Inc.; in some experiments, we used soluble H1 protein (soluble HA), an available influenza product, for comparison, that could be seen as a surrogate if an egg-derived split vaccine. For our antigen presenting cell model, we chose human monocyte-derived macrophages (hMDMs).

First off, we aimed to see how both the H1 VLPs and the soluble HA protein affected the formation of endocytic vesicles. Endocytic vesicles such as clathrin-coated vesicles and caveolin-coated vesicles are generally involved in the endocytosis of proteins or particles (Selby *et al.*, 2017). The resolution obtained with light microscopy techniques is normally not enough to directly distinguish clathrin or caveolin-coated vesicles that measure around 100-200nm or 50-100nm in diameter, respectively (Parkar *et al.*, 2009; Heintzmann and Ficz, 2013). Therefore, transmission electron microscopy (TEM) of ultra-thin resin-embedded sections were used for this approach. Clathrin-coated vesicles can be identified as "bristle-like coated vesicles" (corresponding with "spikes" of clathrin protein), as they were originally described (Roth and Porter, 1964). Caveolin-coated vesicles are typically smaller (than clathrin-coated vesicles) and do not show that characteristic spiky clathrin coat, however, clathrin-coated vesicles shed their coat before fusing with their target membrane (Lemmon, 2001); taking this into account, we termed "uncoated" vesicles to all those vesicles lacking a bristle coat, which include caveolin-coated vesicles but might also include clathrin-coated vesicles that had shed their protein coat or other kinds of vesicles formed through other endocytic processes.

We quantified and classified both clathrin-coated and uncoated vesicles in hMDMs that were stimulated with either H1 VLPs or soluble HA. To reduce the possibility of including in the quantification endocytic vesicles budding from the trans-Golgi network (that would not be involved in endocytosis) (Robinson and Pimpl, 2014), we only considered endocytic vesicles that were within a distance of 2μm from the plasma membrane. We found that the number of clathrin-coated and uncoated vesicles present in hMDMs significantly increased after 15 min and 45 min of incubation with H1 VLPs, in comparison with control cells, that were not incubated with VLPs. Incubation with soluble HA did not result in any significant change in the number of either kind of endocytic vesicles present in the cells. These results indicated that H1 VLPs stimulate the formation of different kinds of endocytic vesicles in hMDMs, suggesting the possibility that the VLPs are internalized via, at least, two kinds of endocytic processes.

To validate that initial hypothesis, we tested how different endocytosis inhibitors affected the internalization of H1 VLPs into hMDMs. As an indirect method to observe internalization of VLPs, we visualized fluorescently immuno-labeled hemagglutinin in hMDMs incubated with H1 VLPs in different conditions, and measured that fluorescence. It is accepted that permeabilization is required for antibody labeling of antigens located inside the cell (Helenius and Simons, 1975; Goldenthal *et al.*, 1985): we intentionally skipped permeabilization of hMDMs in the experiments discussed here, so that only extracellular hemagglutinin would be labeled (and visualized). Based on this protocol, we interpreted the increases in extracellular hemagglutinin fluorescence as an accumulation of H1 VLPs on hMDMs' membrane, and, conversely, the decrease in fluorescence as internalization of H1 VLPs into hMDMs.

First, we visualized how surface hemagglutinin fluorescence changes normally in hMDMs incubated with H1 VLPs. As expected, hemagglutinin does not accumulate, and surface

fluorescence initially decreases over time, indicating that the H1 VLPs are internalized into the hMDMs.

Based on the previous observations suggesting that H1 VLPs upregulate the formation of clathrin-coated vesicles and possibly caveolin-coated vesicles, we used chlorpromazine and genistein to block clathrin-mediated endocytosis and caveolin-dependent endocytosis, respectively. Additionally, we used amiloride as a macropinocytosis inhibitor. Consistently, all three inhibitors resulted in an initial increase in hemagglutinin fluorescence at the surface of hMDMs, and then in a decrease of fluorescence to control values. We interpreted this as a partial effect of all the inhibitors in the internalization of H1 VLPs; these results indicated that CME, caveolin-mediated endocytosis, and macropinocytosis are each involved in the internalization of H1 VLPs into hMDMs.

Internalization via CME is one of the main endocytosis pathways for ligands bound to their receptor (Mettlen *et al.*, 2018). Then, it is not surprising that hMDMs use CME to take up H1 VLPs once they are bound. In fact, influenza typically enters their target cells through this process (Rust *et al.*, 2004). Clathrin-coated vesicles formed during CME deliver their cargo to the early endosome. Generally, the early endosome undergoes acidification and fuses with the lysosome; the acidification process favors protein degradation (Selby *et al.*, 2017). In the case of antigen presentation in antigen-presenting cells, the lysosome has been shown to be involved in MHC II presentation (Michelet *et al.*, 2015). Thus, the fact that H1 VLPs are internalized in part via CME can be an indirect indicator of the later endosomal pathways that would lead to MHC II presentation.

Even though it was initially thought that influenza enters infected cells via CME, later studies confirmed that the virus may enter the cell via other clathrin-independent pathways (Sieczarski *et*

al., 2002). Caveolae, an organelle formed in caveolin-dependent endocytosis pathway by fusion of caveolin-coated vesicles, has been suggested as an alternative internalization route for influenza in epithelial cells (Nunes-Correia *et al.*, 2004). In our discussion of the results, we suggest that, as it has been shown in different cell models with influenza, H1 VLPs are able to utilize caveolin-dependent endocytosis for entry into macrophages. Caveolin-dependent endocytosis-derived compartments are known to not acidify but to remain alkaline (Parton and Howes, 2010). Inhibition of the acidification of intracellular compartments has been shown as a mechanism to (down-) regulate lysosomal antigen degradation in dendritic cells which promotes, in turn, cross-presentation of antigens (Savina *et al.*, 2006; Jancic *et al.*, 2007). The fact that the H1 VLPs use caveolin-dependent endocytosis might favor cross-presentation of antigens on MHC I molecules as an alternative to the classic lysosomal-MHC II presentation.

De Vries *et al.* in 2011 suggested macropinocytosis as yet another alternative route for influenza into epithelial cells. In the present study, we also show macropinocytosis as a pathway of internalization of H1 VLPs into macrophages. Macropinocytosis occurs in antigen presenting cells constitutively as a non-specific antigen uptake process (Lim and Gleeson, 2011). Macropinocytosis has been linked to both MHC I (Norbury *et al.*, 1995) and MHC II (Sallusto *et al.*, 1995) antigen presentation processes. Our experiments show how macropinocytosis is a plausible pathway for H1 VLPs to enter macrophages: this adds to the variety of routes and processes involved in the internalization of these particles.

Finally, when the three mechanisms (CME, caveolin-dependent endocytosis and macropinocytosis) were inhibited at the same time by the addition of dynasore, the hemagglutinin fluorescence at the surface of hMDMs not only increased initially but kept increasing later. We interpreted these results as a major effect of dynasore on the endocytosis of the H1 VLPs,

indicating that CME, caveolin-dependent endocytosis, and macropinocytosis are the main internalization pathways involved in the uptake of H1 VLPs. In addition, dynasore also induced the formation of membrane fragments rich in hemagglutinin fluorescence, which might consist of shed receptor clusters. Receptor shedding is a phenomenon that has been observed in different immune cell types, including T cells and macrophages (Jin *et al.*, 2000): for example, in the presence of LPS, which is known to target and activate macrophages, these cells have been shown to shed different receptors (Leeuwenberg *et al.*, 1994). Furthermore, the binding of an antigen to its cell receptor can induce conformational changes in the receptor that lead to cell activation and/or receptor shedding signalling (Hayashida *et al.*, 2010). Normally, endocytosis of ligands bound to their receptors directs them to the lysosome for degradation, which terminates signalling (Marmor and Yarden, 2004). The accumulation of fluorescence in cellular fragments observed in our study might be the result of receptor shedding events caused by a continuous accumulation of H1 VLPs at the cellular surface that trigger activation and shedding signals; at the same time, the inhibition of endocytic processes by the action of dynasore does not allow for signalling downregulation, resulting in an accentuation of the receptor shedding effect. This also indicates that individual inhibition of CME, caveolin-dependent endocytosis, or macropinocytosis processes can be compensated by the remaining pathways, since these individual inhibitions did not result in massive membrane shedding.

All these findings highlight how heterogeneous the internalization processes of H1 VLPs into hMDMs can be, and offer different possibilities as to how these particles are handled and how the hemagglutinin present on them can be processed via a variety of mechanisms that may lead to antigen presentation via both MHC I and MHC II. This helps explaining the diverse immune response elicited by these particles, which has already been demonstrated by Landry *et al.* in 2014.

Most endocytic vesicles will deliver their cargo to the early endosome, often referred to as the cell's "sorting station" (Jovic *et al.*, 2010): this organelle is in charge of sorting of the endocytosed material for its delivery to different other organelles, involving degradation or recycling of the cargo, depending on the pathway followed (Piper and Katzmann, 2007). We studied how H1 VLPs and soluble HA protein affect the formation of organelles involved in these processes. By electron microscopy, up to 4 different endocytic compartments were distinguished, based in their morphological appearance. First, we quantified early endosomes (EEs), which typically look like empty round vesicles ranging between 500nm and 1μm in diameter, even though different other morphologies (e.g. tubular) are possible (Gruenberg *et al.*, 1989). Secondly, we quantified a subset of EEs whose membranes retain a clathrin-lattice, which are generated by the fusion of clathrin-coated vesicles (Raposo *et al.*, 2001). These clathrin-coated endosomes, or clathrin-coated early endosomes (CCEEs) are early endosomes featuring a dark area on their membrane, corresponding with a clathrin lattice. The third kind of organelle quantified was the multi-vesicular bodies (MVBs), implicated in the lysosomal pathway for protein degradation; as their name indicates, these are early endosomes (vesicles) containing smaller vesicles inside, which form by invagination of the endosomal membrane (Piper and Katzmann, 2007). However, some MVBs quantified do not display internal vesicles clearly, perhaps due to degradation or fusion of these vesicles eventually with the endosomal membrane: thus, we also counted early endosomes showing protein debris inside them as MVBs. The last endosomal compartment quantified was the heterolysosome (HL): HLs are vesicles containing lysosomes. Lysosomes have been described as "electron-dense" organelles in, because of how their membrane looks dark in electron microscopy (Neiss, 1983). HLs can be formed when the late endosome fuse with lysosomes, but also when endocytic vesicles fuse with the lysosome directly (Mego and McQueen, 1967). Thus, it is not

surprising that some of the HLs observed while performing this quantification were smaller than the average early endosome.

We found that the number of EEs present in hMDMs increased significantly after 15 min of incubation with H1 VLPs, and then went down to almost control values at 45 min and 2 hours. Similarly, the number of CCEEs in hMDMs incubated with H1 VLPs for 15 min was significantly higher than in non-exposed cells, and it later decreased. However, the number of MVBs and HLs in hMDMs did not increase or decrease in a significant manner in any of the time points of incubation with H1 VLPs. Incubation of hMDMs with soluble HA did not alter the number of any of the organelles studied significantly, at any incubation time point. These results indicate that the H1 VLPs stimulate the formation of EE and CCEE in hMDMs. This suggests that the H1 VLPs are not only internalized via different routes (as shown earlier) but also being delivered into the early endosome in at least two different ways: via clathrin-coated vesicles into EEs that retain the clathrin protein lattice (becoming CCEEs), and via other mechanisms that deliver to the EE independently of clathrin. However, these experiments didn't provide information about later destinations of the H1 VLPs.

To confirm these initial hypotheses and further explore other intracellular fates of the H1 VLPs and the soluble HA, we used co-localization experiments. To cover different possible destinations of the particles, we analyzed the co-localization of HA and three kinds of endosomal markers: we used EEA1 as an EE marker, and clathrin as a marker for the CCEEs. We referred to these two markers as "early endosomal markers" since they labeled events occurring at the earlier times of endosomal processing. From the early endosome, there are two main pathways that the cargo may follow in the context of antigen presentation. One of these is the late endosomal/lysosomal pathway, where the EE mature and fuse with the lysosome; to analyze the possibility that the HA

present in the studied particles is following this route, we measured the co-localization between HA and LAMP1, a lysosomal marker. The last group of markers analyzed were the "recycling endosomal markers": recycling endosomes form from the early endosome as an alternative to lysosomal degradation. We used Rab3c as a recycling endosomal marker based on previous observations showing this molecule co-localizing with MHC I, highlighting its importance in antigen cross-presentation in recycling compartments. Alternatively, we used Rab4 as a marker for "quickly" recycling endosomes; Rab4 is present in vesicles that recycle material back to the membrane, even before it has been delivered to the early endosomes (Maxfield and McGraw, 2004). Rab4 has not been implicated in MHC I cross-presentation.

The fluorescent signal of HA when it was delivered as soluble HA was consistently low and barely detectable at the 45 min time point of incubation in hMDMs. Thus, for the co-localization analyses, the conditions where hMDMs were incubated with soluble HA were not included. This observation suggests that the soluble HA protein is degraded promptly in hMDMs. Other studies done by our research group also showed that the same soluble HA protein used here is almost exclusively internalized via clathrin-mediated endocytosis and directed to the lysosomal compartment for quick degradation, in antigen presenting cells. This is consistent with the relatively lower cell-mediated immune response generated by influenza vaccination with inactivated split vaccines (Bonduelle *et al.*, 2013).

Co-localization between HA and EEA1 was high when hMDMs were incubated with H1 VLPs for 15 min, and it decreased significantly at 45 min. Similarly, co-localization between HA and clathrin was significantly higher at 15 min than at 45 min, when hMDMs were incubated with H1 VLPs; it is important to highlight that the co-localization value of HA and clathrin are always lower than HA and EEA1, consistent with the concept that only a subset of early endosomes are

clathrin-coated. These results indicate that a great part of HA delivered on H1 VLPs is initially (before 15 min) directed towards EEs, part of which are coated with clathrin, and then it is moved towards other compartments, as indicated by the decrease in co-localization: this suggests that HA in the form of H1 VLPs is first delivered to the EE and then sorted into other different compartments. Also, clathrin-coated regions in CCEEs have been related to protein sorting towards the late endosome (Raposo *et al.*, 2001); this suggests that part of the HA has already been pre-determined to follow the lysosomal degradative pathway at this early (15 min) time point.

Co-localization between HA and LAMP1 was moderate at 15 min but increased significantly after 45 min, in hMDMs incubated with H1 VLPs. This opposite trend in co-localization (as compared to the two previous markers, EEA1 and clathrin) indicates that the major part of HA present on H1 VLPs is initially not located in the lysosome, but it is only at later (45 min) when it predominantly appears in this compartment. These results, together with the previous observation, suggest that part of the HA that is leaving the early endosomal compartment is being delivered, in part, to the lysosome, when H1 VLPs are processed in hMDMs.

Rab3c and HA show some co-localization at 15 min, but the co-localization decreases to almost none at 45 min, in hMDMs incubated with H1 VLPs. This observation indicates that part of the HA on the VLPs is delivered initially to Rab3c-positive recycling compartments, but it is degraded "quickly", or at least before 45 min have passed since HA enters the cell. Normally, for antigen cross-presentation in recycling compartment, proteins that will be loaded on MHC I molecules are degraded rather slowly, since fast degradation is known to inhibit this process (Trombetta and Mellman, 2005). Our results here only show that HA delivered as H1 VLPs remains in Rab3c-positive compartments at 15 min but had almost disappeared from them at 45 min.

Finally, co-localization between HA and Rab4 was moderately high at both time points studied (15 min and 45 min), when the hMDMs were incubated with H1 VLPs. This indicates that HA is continuously present in Rab4-positive compartments. This raises the possibility that some of the HA is continuously being recycled back to the plasma membrane when H1 VLPs enter hMDMs, in a rapid recycling compartment that is not principally involved in antigen presentation. This partial co-localization of HA in with Rab4 accounts for the "missing" co-localization of HA and the other cellular compartment studied at both earlier and later time point. *Per se*, this observation does not seem to have any implication in antigen presentation, but it corroborates the highly diverse nature of the H1 VLPs processing in hMDMs, and it shows how the VLPs are able to be delivered into many intracellular compartments.

Altogether, these results show how the H1 VLPs are diverse not only in terms of how they are internalized by antigen presenting cells, but also how these particles are handled in different ways. H1 VLPs are in part directed towards the late endosome/lysosome for slow degradation: this pathway has been for long known to favor MHC II presentation (Wu *et al.*, 1995). H1 VLPs might also be delivered into Rab3c-positive compartments: Rab3c-has been shown to co-localize with MHC I (Zou *et al.*, 2009), which supports the idea that the VLPs can also be loaded on MHC I molecules. Once again, the intracellular routes that HA follows when delivered as H1 VLPs helps to explain the unique immunogenicity elicited by these particles, shown by Landry *et al.* (2014).

6.2. Conclusions and impact of the findings

The objectives of this project were to identify and understand the internalization routes that VLP utilize in order to enter antigen presenting cells and how they are processed intracellularly, in order to understand how these two processes determine the immunogenicity of the particles.

We found that H1 VLPs are able to enter hMDMs via different routes, namely clathrin-mediated endocytosis, caveolin-dependent endocytosis, and macropinocytosis. We also found that the VLPs are mainly delivered to the early endosomal compartment, where they can be trafficked both towards recycling endosomal compartments and lysosomal compartment.

These findings suggest that the VLPs can be directed towards different degradative compartments where hemagglutinin may be loaded on MHC I, if they are directed to recycling compartments, or also MHC II, if they are directed towards the lysosomal compartment.

Antigen presentation on MHC I molecules leads to CD8+ T lymphocytes activation (Zinkernagel and Doherty, 1974; Zhang and Bevan, 2011). Upon contact with the antigens presented on MHC I molecules on antigen presenting cells, CD8+ T cells proliferate and mount a response to eliminate infected cells (Callan *et al.*, 2000; Yoon *et al.*, 2010). This process has been demonstrated to be key in the control of the expansion of viral infections (Gulzar and Copeland, 2004).

On the other hand, presentation of antigens on MHC II promotes CD4+ T lymphocytes activation (Itano and Jenkins, 2003). A subset of CD4+ T cells are the Th1 cells, which will secrete cytokines that activate phagocytic activity of macrophages and also CD8+ T cells, thus helping in the

elimination of infection (Murray *et al.*, 1985; Kim *et al.*, 2006). Furthermore, other cytokines secreted by Th1 cells promotes the development of memory CD8+ T cells, crucial in the generation of immune memory and ensuring a strong immune response in case of re-exposure to a pathogen (Williams *et al.*, 2009). Another subset of CD4+ T cells are the follicular helper T cells or Tfh cells. They play an essential role in the activation of B lymphocytes, promoting the production of antibodies, and also participate in the generation of memory B cells (Luckheeram *et al.*, 2012).

The fact that the VLPs made by Medicago Inc. can be internalized and processed intracellularly in ways that promote both MHC I presentation to CD8+ T cells and MHC II presentation to CD4+ T cells has very important implications. As mentioned above, the stimulation of different population of T lymphocytes suggests that the VLPs have a strong potential as vaccines, since the activation of these cells are able to promote immune memory and mount immune responses through different mechanisms. The results presented in this thesis help explain how Medicago's VLPs may overcome limitations such as, for example, the limited efficacy in immuno-compromised groups or in the elderly, a recurrent problem in influenza vaccination, where broader and stronger immune response-generating vaccines have been proposed as a solution (Soema *et al.*, 2015).

6.3. Future directions

H1 VLPs have been shown to stimulate endocytic vesicles formation in hMDMs, however, the H1 VLPs were rarely seen inside endocytic vesicles. H1 VLPs seemed to visually disappear promptly after internalization into hMDMs in most experiments conducted. Previous research conducted by our lab showed the structure of the HA contained in H1 VLPs at low resolution and revealed the possibility of better antigen accessibility to the immune system as the HA molecules are well-separated on the VLP surface. Nevertheless, higher resolution is required to understand how the ultrastructural nuances of the plant-expressed HA protein may affect the rapid binding, internalization, and/or fusion of these particles.

Immuno-gold labeling electron microscopy revealed VLP-like structures surrounding the hMDMs membrane, but the VLPs were never seen inside endocytic pits. More defined and finer quantification of the endocytic vesicles stimulated by the VLPs may be required to better characterize this process in a direct manner, without relying on inhibitors and fluorescent labeling of HA, which are indirect methods to determine internalization.

Inhibition assays showed that H1 VLPs may enter the cell via macropinocytosis. This process is well characterized and involves evagination of the cellular membrane to engulf extracellular material. However, these structures were not seen frequently by electron microscopy when analyzing the cellular morphology of hMDMs exposed to H1 VLPs. Additional experiments showing macropinocytosis as a method of internalization of VLPs may be needed to confirm the observations made in this study.

Co-localization analyses were done with different early, late, and recycling endosomal markers, revealing the presence of HA in all of these compartments at different time points. Additional

markers of the different compartments could be used to confirm and validate the findings. In fact, other studies conducted by our lab and collaborators showed similar trends in co-localization of HA with different markers for early, late, and recycling compartments: in these studies, Rab5 was used as an alternative marker for early endosomes; Rab7 was used as a marker for late endosomes, indicating the presence of HA in late endosome/lysosome route; and Rab11 was used as an alternative for recycling endosomal compartment.

The presence of HA was confirmed in lysosomes and recycling endosomes, suggesting the possibility that HA is degraded in these compartments for MHC II and MHC I presentation, respectively. However, co-localization of HA and MHC molecules was not shown in this study. Our lab showed in collaborative studies with another lab that HA does co-localize with these molecules. Different research groups working with the same vaccine product (H1 VLPs) are currently exploring MHC I and MHC II bound peptides derived from the HA present in the VLPs.

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