Sede Amministrativa: Università degli Studi di Padova

Dipartimento di SCIENZE BIOMEDICHE SPERIMENTALI

SCUOLA DI DOTTORATO DI RICERCA IN BIOSCIENZE
INDIRIZZO BIOLOGIA CELLULARE
XXII CICLO

Comparison of the CD4+ T cell and antibody responses to different Influenza vaccination regimens and to infection in naive and pre-exposed mice

Direttore della Scuola : Ch.mo Prof. Tullio Pozzan
Coordinatore d'indirizzo: Ch.mo Prof. Cesare Montecucco
Supervisore :Ch.mo Prof. Cesare Montecucco

Dottorando : Vanessa Ronconi

INDEX

1. INTRODUCTION

1.1 Influenza

1.1.1 Disease and epidemiology

The term influenza has its origins in the 15th century in Italy, where the cause of the disease was ascribed to unfavourable astrological influences. The word later came to designate, in all languages, human and animal pulmonary infections resulting from epidemic or pandemic viral attacks. In English, it is commonly abbreviated to 'Flu'.

The infectious disease called 'Flu' is an acute contagious respiratory illness caused by influenza viruses. It is one of the oldest and most common diseases known to man and it can also be one of the deadliest. Mention of Flu epidemics can be found as far back as the third century B.C. in a text by Hippocrates, the father of European medicine [1 and 2]. The first well-described influenza pandemic was recorded in 1580; from the 16th century, a study of historical archives indicates an average of three serious epidemics or pandemics per century.

Humanity first understood the potential danger of influenza in 1918–1919, when a disease that seemed to come out of nowhere killed over 40 million people around the globe, many more than died in World War I [3]. The highly contagious nature of the resulting lethal pneumonia was obvious, but the first human influenza A virus (**Fig. 1**) was not isolated until 1933 (in ferrets) [4].

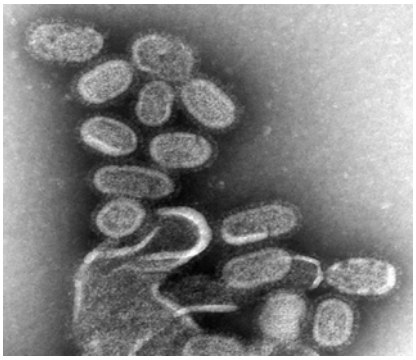


Fig. 1: TEM (Transmission Electron Microscopy) of negatively stained influenza virions, magnified approximately 100,000 times. Figure from the Centers for Disease Control and Prevention's Public Health Image Library.

New influenza A viruses caused human pandemics in 1957 and 1968 [5], and regular epidemics result from antigenically 'drifted' influenza strains.

Worldwide monitoring of influenza viruses through surveillance is the mechanism by which the evolution of circulating viruses can be monitored. The Geneva-based World Health Organization (WHO) influenza surveillance network (GISN) was established in 1952 (**Fig. 2**); it links four collaborating centers (London, Tokyo, Melbourne and Atlanta) and 128 National Influenza Centres in 99 countries to conduct the necessary surveillance and provide the WHO with the information it required to advise its member states on the most effective influenza control measures.

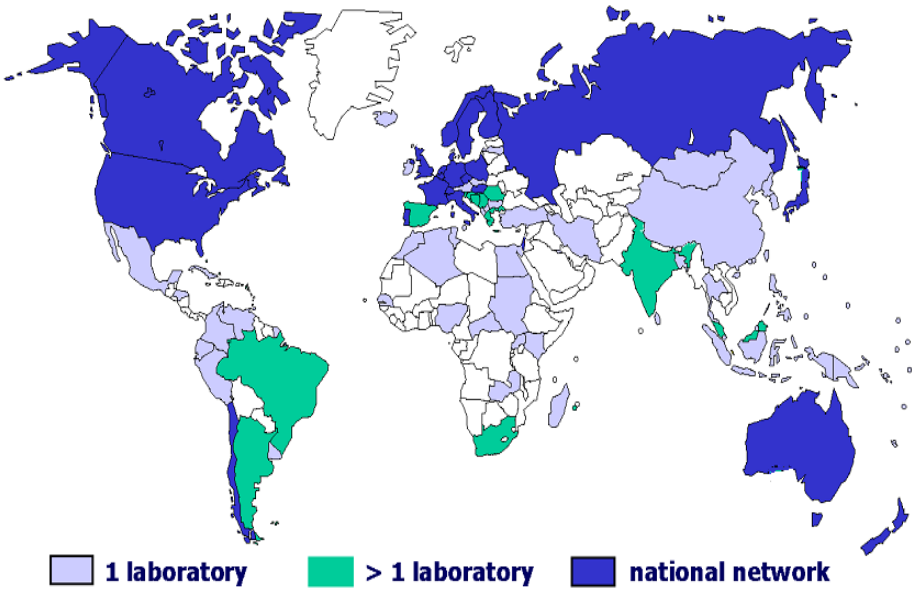


Fig. 2: WHO Influenza Surveillance Network.

Influenza is a highly contagious viral disease of the respiratory tract. Although most influenza infections are self-limited, few other diseases exert such a huge toll of suffering and economic loss [6]. About 20% of children and 5% of adults worldwide develop symptomatic influenza each year [7]. It causes a broad range of illness, from symptomless infection through various respiratory syndromes, disorders affecting the lung, heart, brain, liver, kidneys, and muscles, to fulminant primary viral and secondary bacterial pneumonia. In fact, the disease damages the linings of the respiratory tract, thus secondary bacterial infections, such as pneumonia, meningitis, sinus and ear infections, can then take hold. The course is affected by the patient's age, the degree of pre-existing immunity, the properties of the virus, smoking, comorbidities, immunosuppression, and pregnancy [6].
The main way by which influenza viruses spread is from person to person in respiratory droplets of coughs and sneezes.
In the Northern Hemisphere, seasonal epidemics of influenza generally occur during the winter months on an annual or near annual basis and are responsible for approximately 36,000 deaths in the United States each year [4 and 8]. Influenza virus infections cause disease in all age groups, but rates of serious morbidity and mortality are highest among the elderly, infants and those people of any age who have medical conditions that place them at high risk for complications from influenza (e.g., chronic cardiopulmonary disease).

1.1.2 Virus

Structure and classification

Influenza viruses are enveloped ribonucleic acid (RNA) viruses with a segmented genome belonging to the family of *Orthomyxoviridae* [9].
They are divided into types A, B, and C according to the structural (matrix [M] and nucleoprotein [NP]) genes. Of these, influenza A and B viruses cause annual epidemics. Humans are the only hosts for influenza B viruses, but influenza A viruses infect a variety of species, including birds, pigs, horses, dogs, and humans [4 and 10].
Both influenza A and B viruses contain 8 RNA segments, each individually encapsidated by the viral nucleoprotein, and possess two surface glycoproteins embedded into the

membrane: the haemagglutinin (HA) and neuraminidase (NA), which are able to elicit antibody responses in humans [9].

The nomenclature for influenza viruses includes the virus type, the geographic site of isolation, a serial number and the year of isolation. In addition, influenza A viruses are classified into subtypes according to the two surface antigens HA and NA (**Fig. 3**). Currently, there are 16 known HA and 9 known NA subtypes [11].

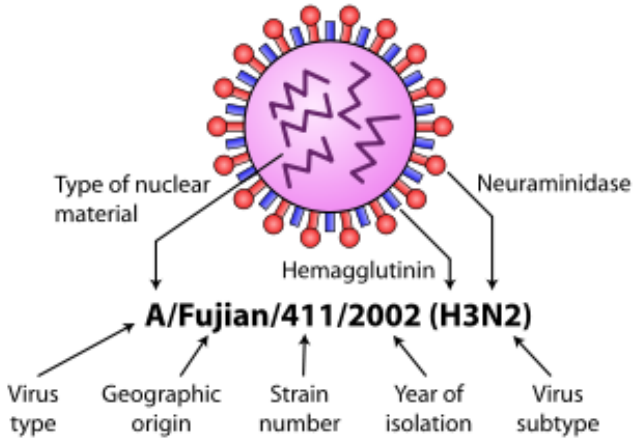


Fig. 3: Diagram of influenza virus nomenclature. Figure from Wikipedia, the free encyclopedia.

The structure of the influenza virus (**Fig. 4a**) is somewhat variable, but the virion particles are usually spherical or ovoid in shape and 80 to 120 nanometers in diameter, although filamentous forms can occur. The influenza virion is an enveloped virus that derives its lipid bilayer from the plasma membrane of a host cell. The two major spike glycoproteins, HA and NA, are incorporated into the virus envelope during budding, together with much lesser amounts of the M2 proton-selective ion channel protein [12]. Haemagglutinin binds to sialic acid-containing receptors on the cell surface, is responsible for the penetration of the virus into the cell cytoplasm by mediating low pH-induced fusion between the virus and endosomal membranes, and is the major viral antigen against which neutralizing antibodies are generated [12]. The neuraminidase enzyme catalyses the cleavage of viral progeny from infected cells. It also facilitates the movement of the virus through inhibitory mucopolysaccharides coating the respiratory

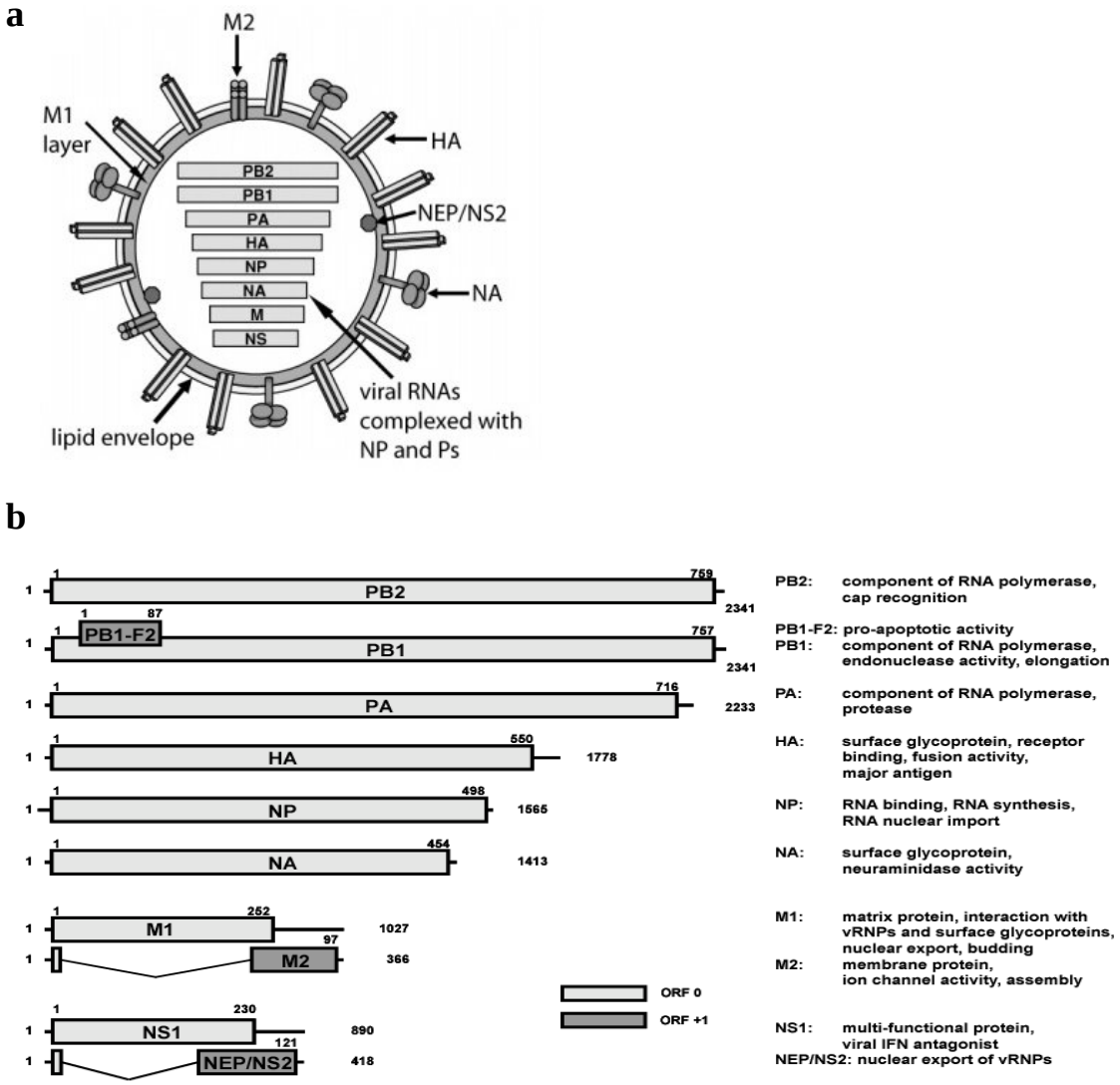


Fig. 4: Schematic of an influenza A virus. Fig. 4a shows a structural diagram of influenza virus. In **Fig. 4b** there is a diagrammatic representation of influenza A virus showing protein and RNA composition. Figures from "Fields VIROLOGY", vol. 1, chapter 46, 5th Edition. *Orthomyxoviridae*: The Viruses and Their Replication (Peter Palese and Megan L. Shaw).

tract epithelium, allowing cell to cell spread through the respiratory mucosa [9].

The third surface protein, the M2 ion channel, is a tetrameric membrane channel important in the regulation of the internal pH of the virion. By conducting protons from the acidified endosomes into the interior of the virus, the M2 ion channel is essential for mediating the dissociation of the ribonucleoprotein complex from the rest of the viral components during the virus uncoating process.

On the inner side of the envelope that surrounds the influenza virion there is an antigenic matrix protein lining (M1). Within the envelope there is the influenza genome, which is organized into 8 segments of negative-sense RNA (A and B forms only; influenza C has 7

RNA segments); each RNA segment codes for one or two proteins. For example, the influenza A genome comprises 8 segments of RNA, encoding for 11 proteins: haemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), M1, M2, NS1, NS2 (NEP), PA, PB1, PB1-F2 and PB2 (**Fig. 4b**). Two RNAs (the M1 and NS1 genes) give rise to a spliced mRNA encoding the M2 and the NEP/NS2 proteins, respectively; instead, the PB1-F2 protein is encoded by an open reading frame overlapping the PB1 open reading frame. The RNA is packaged with nucleoprotein into a helical ribonucleoprotein form, with three polymerase proteins for each RNA segment.

Replication cycle of influenza virus

The virus particle initially associates with a human host cell by binding to sialic acid-containing receptors on the host cell surface with its haemagglutinin (step 1 in **Fig. 5**) [13]. The cell imports the virus by endocytosis. The acidic environment of the endosome induces a conformational change in the HA protein and this leads to viral membrane fusion with the vacuole's membrane, releasing the uncoated viral ribonucleoprotein complex and RNA-dependent RNA polymerase into the cytosol of the host cell (step 2) [14]. The ribonucleoprotein complex is transported through the nuclear pore into the cell nucleus. Once in the nucleus, the incoming negative-sense viral RNA (vRNA) is transcribed into messenger RNA (mRNA) by a primer-dependent mechanism (step 3a). The replication occurs via a two step process: a full-length complementary RNA (cRNA), a positive-sense copy of the vRNA, is first made via a primer-independent mechanism and this in turn is used as a template to produce more vRNA (step 3b). The mRNAs are exported into the cytoplasm and translated (step 4). Newly-synthesized viral proteins are either secreted through the Golgi apparatus onto the cell surface (in the case of neuraminidase and haemagglutinin, step 5b) or transported back into the nucleus to bind vRNAs and form new viral genome particles (step 5a).

Negative-sense vRNAs that form the genomes of future viruses, RNA-dependent RNA polymerase, and other viral proteins are assembled into viral particles and leave the nucleus. Haemagglutinin and neuraminidase molecules cluster into a bulge in the cell membrane. The vRNA and viral core proteins enter this membrane protrusion (step 6) and the mature virus buds from the host cell enveloped in the host cell's membrane (step 7)

[15]. Influenza virus particles have to be actively released because the HA anchors the virus to the cell by binding to sialic acid-containing receptors on the cell surface. The enzymatic activity of the neuraminidase protein is required to remove the sialic acid from the surface of the cell as well as from the virus particles and allows the virus to leave its host cell [13]. After the release the new viral particles are able to invade other cells.

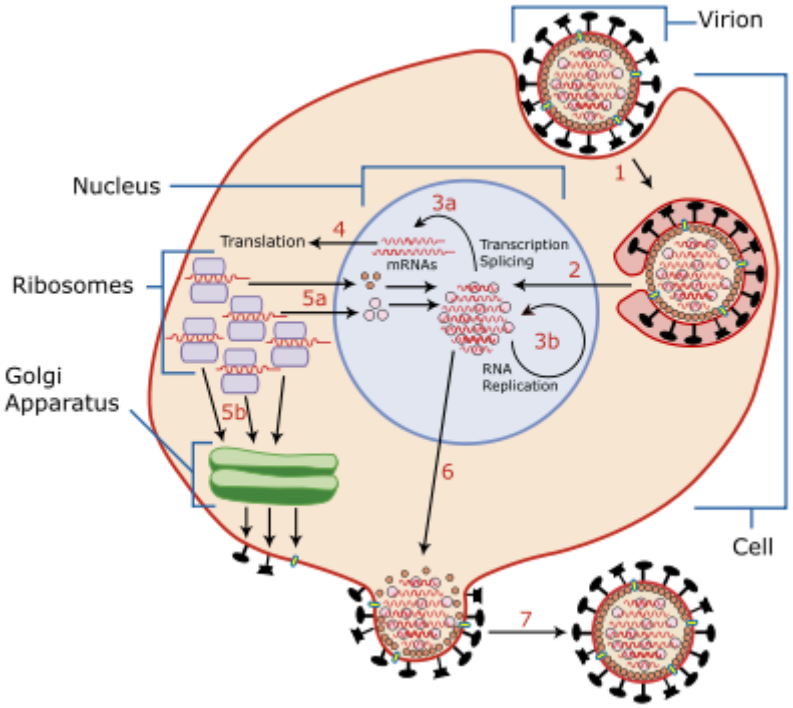


Fig. 5: Influenza virus replication cycle (the steps in this process are discussed in the text). Figure from Wikipedia, the free encyclopedia.

Antigenic drift and shift

All successful viruses evolve strategies to ensure their survival in nature, which in turn determine the pathogenesis and outcome of the associated infectious process [3].

Influenza viruses continuously undergo antigenic change to escape the host's acquired immunity. Two distinct mechanisms of antigenic evolution can be identified: antigenic drift causes regular influenza epidemics, while antigenic shift is the cause of occasional global outbreaks of influenza (pandemics).

Antigenic drift is the accumulation of mutations in all influenza gene segments, but the changes are particularly important in the surface glycoproteins (HA and NA), which are constantly subject to selection pressure by the host's defense mechanism. The process of antigenic drift is subtle and gradual, involving point mutations within antibody-binding sites in the HA protein, the NA protein, or both, which potentially occur each time the virus replicates [16, 17, 18 and 19]. In fact, the point mutations are caused by the inherent error rate of the RNA-dependent polymerase complex, which lacks proofreading ability [20]. Most of these mutations are 'neutral' as they do not affect the conformation of the proteins; however, some mutations cause changes to the viral proteins such that the binding of host antibodies is affected. Consequently, infecting viruses can no longer be inhibited effectively by host antibodies raised to previously circulating strains, allowing the virus to spread more rapidly among the population [21]. This causes the seasonal Flu epidemics. The seasonal Flu viruses make slight changes all the time and this is why sequential variants of the same virus circulate for decades [5]. The influenza vaccine has to be reformulated almost every year to take account of the changing virus.
Antigenic drift occurs in all strains of A and B viruses, although the observed evolutionary patterns vary dependent on the strain.

Antigenic shift, which is seen only with influenza A virus, occurs at infrequent and unpredictable intervals, when the current influenza A virus disappears and is replaced by a new subtype with novel glycoproteins (always a novel HA and often a novel NA); the source of such viruses is thought to be other mammals and birds [22]. Antigenic shift occurs as a result of genetic reassortment of the genome segments from different influenza A viruses in a doubly infected host cell (**Fig. 6**). Although 3 HA proteins and 2 NA proteins have dominated human influenza infections over the past 100 years, 16 different influenza A virus HA types and 9 distinct NA types are now circulating in nature, infecting species as diverse as ducks, pigs, leopards, seals and even whales [4]. This raises the possibility that a new influenza A virus may enter the human population at any time as a consequence of the reassortment process known as antigenic shift [3]. There is little or no background immunity in the population to the new virus (antibodies towards the previously circulating subtype do not cross-react with the new subtype) so it spreads rapidly, usually causing pandemics with extensive morbidity and mortality [20].

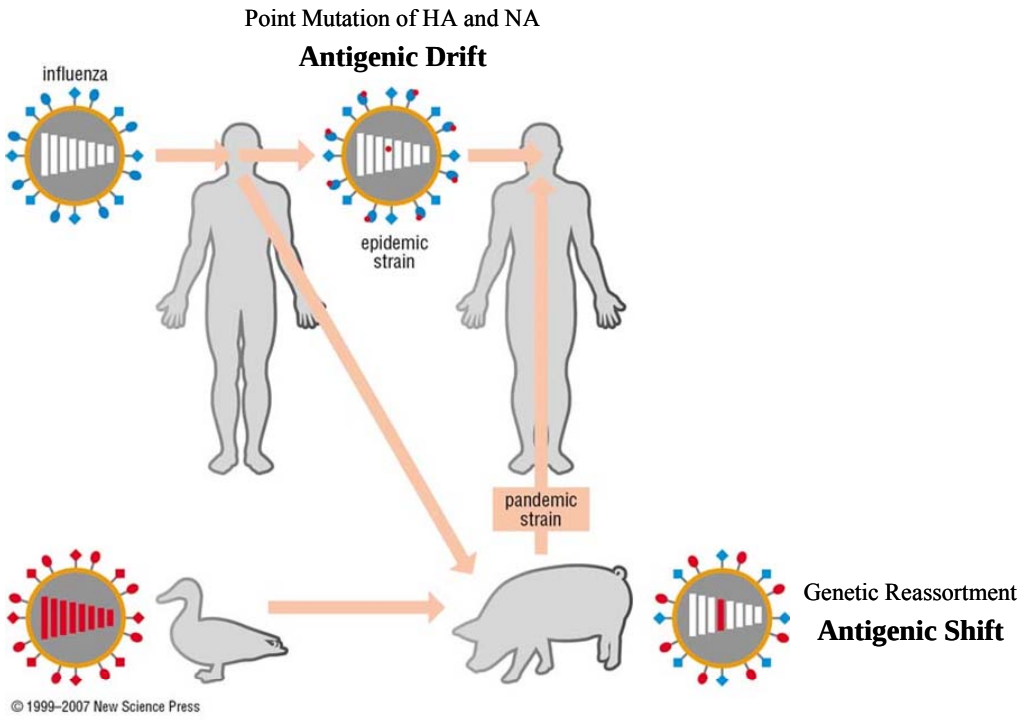


Fig. 6: Antigenic drift and shift underlie influenza epidemics and pandemics, respectively. Figure adapted from Immunity: The Immune Response in Infectious and Inflammatory Disease (DeFranco, Locksley and Robertson).

1.2 Immune response to viral infection

The mammalian immune system is comprised of two arms: innate and adaptive immunity. The innate immune system is the first line of host defense against pathogens providing non-specific microbial killing mediated by leukocytes and by the complement system. The adaptive immunity is involved in elimination of pathogens in the late phase of infection as well as the generation of immunological memory and is mediated by antigen-specific lymphocytes [23].

The host defense against viral infection consists of a complex interplay between components of the innate and the adaptive immune system. The innate immune response is often insufficient by itself to clear or permanently control viral infections; however, it plays a very important role in keeping the virus load low until a specific adaptive immune response can be generated. Equally important, components of the innate response shape the adaptive immune response and direct the subsequent effector phase [24].

The innate immune system comprises several cell types, including dendritic cells (DCs), monocytes/macrophages and natural killer (NK) cells. They, besides constraining the virus spread and eliminating virus-infected cells, secrete high levels of proinflammatory cytokines and chemokines, which direct not only the activation and differentiation of the adaptive immune response but also the subsequent recruitment of antigen-primed effector T cells to the sites of viral replication. The adaptive immune response towards viruses is mediated by T and B lymphocytes, which are activated and differentiate in the secondary lymphoid organs through an interplay between several cell types of both the innate and the adaptive immune system, including professional antigen-presenting cells (APCs). DCs are a specialized family of APCs that effectively link innate recognition of invading pathogens to the generation of the appropriate type of adaptive immune response [25]. During viral infection, they play a key role in sensing and processing viral proteins and directing the differentiation of naïve T and B cells to virus-specific effector T cells and antibody-secreting plasma cells. Following their activation in the peripheral sites, the activated DCs migrate towards the regional draining lymph nodes, where they present the antigen to rare antigen-specific T and B cells that eventually become activated and may interact to regulate the induced adaptive responses [25, 26 and 27].

1.2.1 Innate immunity and influenza virus infection

Innate immunity refers to host defense functions that are directly encoded in the genome and are capable of recognizing and inhibiting infectious agents [28].

After initial exposure to a novel influenza viral strain, it takes between 5 and 7 days before specific antibodies and T cells arrive in the lung to definitively clear the virus; hence, this defines the time window in which innate immunity is critical. In most cases, influenza viruses remain confined to the upper respiratory tract in humans, despite the ability of the virus to bind to most cells. The innate immune mechanisms, by developing very quickly and controlling virus replication during the early stages of infection, are critical in restricting the anatomic spread of influenza virus and facilitating the rapid development of adaptive responses [28].

Numerous host defense mechanisms, including mechanical barriers, block respiratory tract infection. The respiratory tract is covered with a mucociliary layer consisting of ciliated

cells, mucus-secreting cells and glands. Foreign particles in the nasal cavity or upper respiratory tract are trapped in mucus, carried back to the throat, and swallowed.
Upper respiratory tract **epithelial cells** are the first target cells for influenza virus infection and the first cells to mount an innate response. Binding of influenza viruses to respiratory epithelial cells, as to other cells, is mediated by attachment of the HA to sialic acids expressed on proteins or lipids on the surface of the cells [29, 30, 31 and 32].
Infection of respiratory epithelial cells with influenza results in release of type 1 interferons (IFNα/β) and chemokines that promote the recruitment of neutrophils. Influenza is a lytic infection leading to death of respiratory epithelial cells. Apoptotic and necrotic epithelial cells then release factors that promote phagocytosis [33] by macrophages or neutrophils and recruitment of neutrophils [34]. The respiratory epithelium can also release defensins and surfactant proteins that can contribute to antiviral defense [35]. Hence, it is appropriate to view the respiratory epithelial cells as immune response cells.

Many cells of the innate immune system express germline-encoded pattern recognition receptors (PRRs), which recognize molecular patterns conserved through evolution in a wide range of pathogens (pathogen-associated molecular patterns, PAMPs) [36]. The best described of the PRRs are those of the Toll-like receptor (TLR) family. Influenza virus infection is recognized by the innate immune system through TLR7, which recognizes influenza genomic RNA upon release in late endosomes, [37 and 38] and retinoic acid inducible gene I (RIG-I), which detects viral single stranded RNA in the cytoplasm after fusion and replication in infected cells [39].
The innate recognition of viral components through PRRs leads to a program of gene expression that promotes a localized antiviral state and elicits the recruitment of inflammatory cells to the site of infection by the secretion of proinflammatory cytokines and chemokines that coordinate innate and adaptive immunity. Multiple cytokines and chemokines are produced by influenza virus-infected epithelial cells, macrophages and DCs. The major inflammatory cytokines of the innate response induced by a viral infection include TNFα, type I IFNs, IL-1, IL-6, IL-12 and IL-18, where **type I IFNs** represent one of the most critical mediators in the activation of innate and adaptive immunity during influenza virus infection [40]: they promote the production of several intracellular antiviral proteins that interfere with virus replication; they induce the recruitment of monocytes/macrophages, NK cells and T cells; they support the maturation

of antigen-presenting cells (APCs) and are important cofactors in the development of the adaptive immune response [41].

Neutrophils infiltrate tissues early after viral infection and, through the expression of proinflammatory cytokines and chemokines, can direct the subsequent recruitment of monocytes and lymphocytes. The initial influx of neutrophils is probably triggered by specific signals released by respiratory epithelial cells and DCs. However, the role of neutrophils in viral clearance or pathogenesis is not yet fully clear.

Alveolar macrophages are important both in killing infectious organisms that reach the lower airways and in release of chemokines and cytokines that have proinflammatory effects and recruit other cells to the lung [28]. In the lung they are present in the interstitium and in the alveoli and are the most numerous phagocytic cells in the uninflamed lung. Because of their physical location, they represent the first line of defense to inhaled pathogens [42]. Blood monocytes or macrophages derived from blood monocytes recruited to the lung also participate in the early host response to influenza. An important beneficial activity of macrophages during influenza infection is the phagocytosis of virus-infected cells, as clearance of apoptotic host cells is essential to limit virus spread [33 and 43].

NK cells are another innate effector cell type that influences the shape of both the innate and the adaptive immune response during viral infection. The effector functions of activated NK cells include lysis of virus-infected cells and the secretion of proinflammatory cytokines and chemokines [44]. In addition, NK cells express activating immunoglobulin natural cytotoxicity receptors, including CD16, which mediate antibody-dependent cellular cytotoxicity (ADCC), eliminating virus-infected cells coated with specific IgG [45]. NK cells are primarly activated by type I IFNs or proinflammatory cytokines mainly produced by DCs [46-50]. In turn, IFNγ and TNFα produced by NK cells can also affect the maturation and the effector functions of neighbouring DCs (up-regulation of co-stimulatory molecules and secretion of cytokines) [51 and 52], as well as other leucocytes, including macrophages, granulocytes and other lymphocytes, which are recruited as a consequence of viral infection. NK cells can also be activated through direct recognition of haemagglutinins on virus-infected cells by NKp46 receptor [24]. This is due to their expression of a sophisticated repertoire of receptors including NKG2D, which recognize an array of cellular stress-induced molecules. Through these receptors, NK cells are able to distinguish between uninfected normal cells and stressed infected cells [53 and 54]. According to the missing self-hypothesis, NK cells may also be activated by the loss

of MHC expression on some infected cells. A set of inhibitory receptors normally recognize MHC class I and block target cell killing, but if the signal from these receptors is too weak, degranulation and cell killing can be induced. In this manner, viruses trying to avoid CD8+ T cell-mediated killing through down-regulation of MHC class I may still be targeted by the host response. However, in the case of influenza virus, after the infection the binding of the two NK inhibitory receptors, KIR2DL1 and the LIR1, to the infected cells is specifically increased. It has been demonstrated that, after influenza virus infection, MHC class I proteins redistribute on the cell surface. Such redistribution allows better recognition by the NK inhibitory receptors and consequently increases resistance to NK cell attack [55].

By expressing a wide array of PRRs shared with cells of the innate immune response, and at the same time displaying the potential to process and present antigens to naïve T cells, **DCs** bridge innate and adaptive immunity. After recognizing foreign antigens in the periphery of the body, DCs undergo maturation and migrate via afferent lymphatics into the draining lymph nodes, where they can induce antigen-specific protective CD8+ CTL responses, as well as CD4+ T helper cell activation, that enforce cellular and humoral immunity [56]. The phenotypic changes that occur in maturation include the upregulation of major histocompatibility complex (MHC) class II and costimulatory molecules and the release of proinflammatory cytokines and chemokines that enhance the DCs' ability to stimulate T cells, leading to the initiation of adaptive immune responses specific for the infecting pathogen [57-59] addition to their critical role in initiating adaptive immune responses, a DC subpopulation called plasmacytoid dendritic cells (pDCs) contribute to the antiviral innate immune system by secreting IFN-α/β, a powerful antiviral cytokine, in response to viral infection [60

1.2.2 Adaptive immunity and influenza virus infection

Most viral infections are controlled by the innate immune system. However, if viral replication overcomes innate defenses, the adaptive response must be mobilized.
The adaptive defense consists of antibodies and lymphocytes, B and T cells, which are involved in the humoral and the cell-mediated responses, respectively.

The ability to shape the response in a virus-specific manner depends upon communication between the innate and adaptive systems. This communication is carried out by cytokines that bind to cells, and by cell-cell interactions between DCs and lymphocytes in lymph nodes.

Circulating mature T cells that have not encountered their specific antigens are called naïve T cells; to participate in an adaptive immune response, a naïve T cell must first encounter an APC that presents to the T cell receptor (TCR) a specific epitope, through specialized host-cell glycoproteins encoded in a large cluster of genes called major histocompatibility complex (MHC). After priming by APCs, naïve T cells can proliferate and differentiate into **effector T cells**. T cells fall into two classes, **CD4+** and **CD8+** T cells that recognize peptide antigens derived from different types of pathogen. Naïve CD4+ and CD8+ cells within lymphoid tissues continually scan the surface of DCs for the presence of cognate antigen/MHC complexes [61]. The recognition of the MHC class I-peptide complexes by the TCR of CD8+ T cells leads to their activation, proliferation and acquisition of the ability to kill infected cells. The MHC class II-peptide complexes are recognized by the TCR of CD4+ T cells and the activation of these cells triggers regulatory mechanisms which orient the immune response. CD4+ T cells are important in regulating immune responses to infection and, depending on the type of antigen encountered and the microenvironment where the antigen is presented to the antigen-specific CD4+ T cell, the immune response may differ dramatically. Upon antigen encounter in the presence of IL-12 and IFNγ, naïve CD4+ cells can differentiate into T helper 1 (Th1) cells that are characterized by the production of proinflammatory cytokines such as IFNγ and mediate cellular immune responses; IFNγ produced by Th1 cells activates macrophages, induces IgG2a (in mice) antibody production by B cells and amplifies the Th1 development, also by inhibiting the proliferation of Th2 cells. Alternatively, antigen signaling in the presence of IL-4 induces the naïve CD4+ cell population to develop into Th2 effectors secreting IL-4, IL-5 and IL-13. These Th2 cells preferentially drive B cells to produce IgG1 and IgE antibodies [62].
Viral infections such as that with influenza A virus generally produce a cytokine milieu that favors the generation of a Th1 or type 1 immunity (**Fig. 7**) that promotes the activation of CD8+ T cells and macrophages [63]. The immune response to influenza is also characterized by the production of a robust antibody response, in particular of the IgG2a subtype, as well as IgA [64]. In addition to their helper functions (by helping B

cells to produce antibodies and activating cytotoxic T lymphocytes and macrophages), CD4+ T cells can also act as direct effector cells by releasing anti-viral cytokines (IFNγ and TNFα) and, as it has been recently reported [65], by a direct cytolytic activity on infected cells.

The continual migration of effector T cells from lymphoid tissues during an acute infection results in a massive increase in the numbers of antigen-specific cells in the lung airways and lung parenchyma from days 7–10 post-infection [66]. The arrival of effector T cells has an immediate and dramatic impact on the viral load through the expression of cytokines and the direct lysis of the infected cells. Influenza-specific CD4+ and CD8+ effector T cells in the lung predominantly produce IFNγ and TNFα. CD8+ effector T cells (cytotoxic T lymphocytes, CTL) play a major role in the clearance of influenza virus from the lungs by inducing the apoptosis of infected epithelial cells through Fas-FasL interactions or the exocytosis of cytolytic granules containing perforin and granzymes [67 and 68].

B cell responses and **virus-specific antibodies** play an important role in the clearance of influenza virus. Studies with B cell-deficient mice have shown that these mice fail to clear the virus and ultimately succumb to infection [69 and 70].

Similarly to T cells, B cells that have not encountered the antigen are called naïve B cells. B cell activation requires both the binding of the antigen by the B cell surface immunoglobulin, the B cell receptor (BCR), and the interaction of B cell with antigen-specific CD4+ T cells. In the secondary lymphoid tissues naïve B cells recognize opsonized antigens on APCs, such as migrating DCs or resident follicular dendritic cells (FDC), by the formation of an immunological synapse and this, together with follicular helper T cells (T_{FH}), drives rapid B cell proliferation. T_{FH} cells are a class of helper T cells specialized in the cognate control of antigen-specific B cell immunity. The direct contact between antigen-specific CD4+ T cells and antigen-presenting B cells in lymphoid tissues, in addition to CD40-CD40L interactions and cytokine signaling, drives B cell proliferation and directs the differentiation of the clonally expanded progeny of the naïve B cells into antibody secreting plasma cells. CD4+ T cells also direct the isotype switching of antibodies produced by plasma cells [71].

Viral infection activates a humoral immune response that is characterized by an early rise of antigen-specific IgM followed by affinity maturation, isotype switching, and the ensuing rise in antigen-specific IgG and IgA antibodies. Th1 immune response, induced

by the influenza virus infection, is associated mainly with the production of IgG2a subclass (in mice; its homologue in humans is IgG1). In addition to its neutralizing activity, it is known that IgG2a is the most efficient isotype at fixing complement and in inducing ADCC mediated by NK cells [72 and 73].

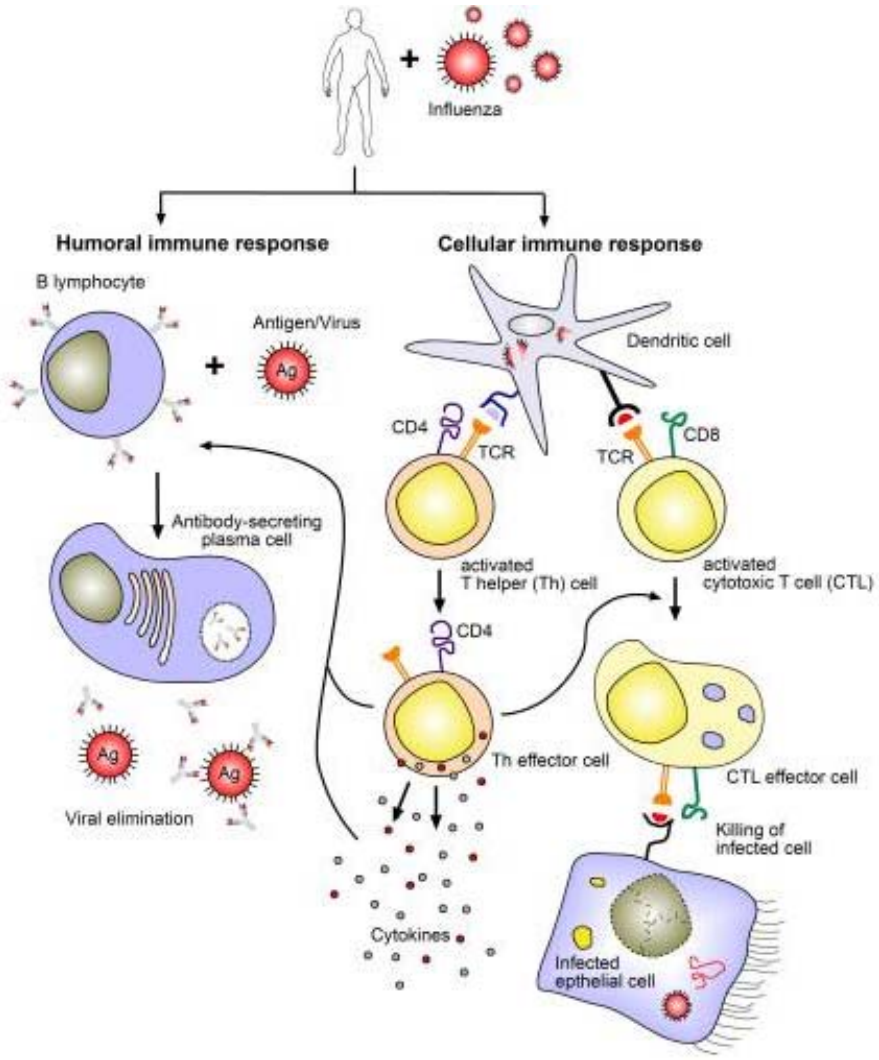


Fig. 7: The humoral and cell-mediated immune response to influenza virus infection. Figure from "Influenza Report 2006", www.InfluenzaReport.com. Chapter 4: Pathogenesis and Immunology (Georg Behrens and Matthias Stoll).

Antibody-mediated protection against infectious agents is based on both direct and indirect functions. The direct function of antibodies, neutralization, refers to the abolition of a pathogen's infectivity upon antibody binding with no participation of any other component of the innate or adaptive immune system. Neutralization is probably the most

powerful function that antibodies exert against viruses, but is not the only one (**Fig. 8**). "Non-neutralizing" antibodies are unable to directly inhibit free virus entry into target cells, but nonetheless exhibit antiviral activity mediated by the Fc region of the antibody molecule. These antibody effector mechanisms include the activation of the complement cascade, which results in the lysis of the virus infected cells, and the NK-mediated ADCC. ADCC depends on interaction of antibodies of the IgG1 and IgG3 subclasses (in humans) bound on the surface of infected cells with the Fc receptor (CD16) expressed on NK cells [74].

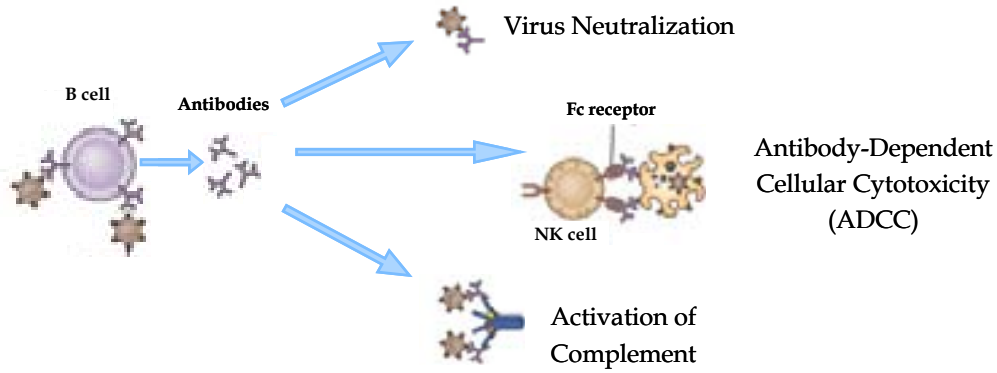


Fig. 8: Mechanisms of action of the antiviral antibodies.

Effector lymphocytes have only a limited life-span and, once antigen is removed, most of the antigen-specific cells generated by the clonal expansion of small lymphocytes undergo apoptosis. However, some persist after the antigen has been eliminated. These cells are known as memory cells and form the basis of immunological memory, which provides lasting protective immunity. Protective immunity against reinfection is one of the most important consequences of adaptive immunity and it depends not only on pre-formed antibodies and effector T cells, but most importantly on the establishment of a population of lymphocytes that mediates long lived immunological memory. The first adaptive response against a virus, called the primary response, often takes days to mature. In contrast, a memory response develops within hours of infection. Memory is maintained by a subset of B and T lymphocytes called memory cells which survive for years in the body and remain ready to respond rapidly and efficiently to a subsequent encounter with the virus. The capacity of these cells to respond rapidly to restimulation with the same

pathogen can be transferred to naïve recipients by primed B and T cells [75]. This so-called secondary response is always stronger than the primary response to infection.

1.3 Vaccine

1.3.1 Influenza vaccines

Vaccination remains the principal measure to prevent seasonal influenza and reduce associated morbidity and mortality.

To prevent seasonal influenza, the Centers for Disease Control and Prevention recommend annual influenza vaccination. Antibodies directed against the influenza haemagglutinin and neuraminidase, which are induced by natural infection or vaccination, have an important role in protection [76]. Most adults and older children have pre-existing levels of antibodies because of prior infection or vaccination [77 and 78]. However, antigenic drift of influenza virus, which is caused by an accumulation of point mutations in the HA and NA genes, occurs both in influenza A and B viruses [79-81]. An individual who was infected by or previously vaccinated against influenza viruses circulating in prior years may still be susceptible to a new virus strain. Therefore, influenza vaccines are reformulated each year based on the results of international surveillance that predict the virus strains that will circulate in a subsequent year [76].

Two classes of influenza vaccines are licensed for interpandemic influenza: inactivated trivalent influenza vaccine (TIV) and live attenuated influenza vaccine (LAIV) [76]. Both types of vaccines are trivalent and contain an influenza A H1N1 subtype virus, an influenza A H3N2 subtype virus, and an influenza B virus to protect against each of the co-circulating strains of influenza [82].

Several types of inactivated vaccines are being used: (i) “whole inactivated virus vaccines”, (ii) “split vaccines” and (iii) “subunit vaccines” (**Fig. 9**). Early whole virus vaccines were associated with frequent local and systemic adverse effects, therefore they are little used and unlicensed in many countries [9]. Split vaccines are produced from the disrupted highly-purified influenza virus. For the preparation of subunit vaccines, the surface glycoproteins HA and NA— important for the induction of virus neutralizing

antibodies and protective immunity — are purified by removing other viral proteins and lipids after disruption of the virus with detergent [83].

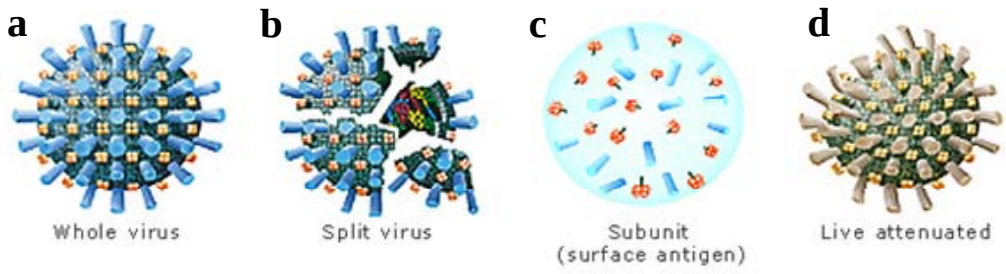


Fig. 9: The currently available licensed seasonal influenza vaccines:
a_ Whole virus vaccines consisting of inactivated viruses.
b_ Split virus vaccines consisting of inactivated virus particles disrupted by detergent treatment.
c_ Subunit or surface antigen vaccines consisting essentially of purified HA and NA.
d_ Live attenuated (cold-adapted) virus vaccines consisting of weakened (non-pathogenic) whole virus.
Figure from: http://www.ifpma.org/Influenza/index.aspx?44.

Successful use of the currently licensed inactivated vaccines to prevent influenza requires close antigenic matching between the circulating viruses and the antigens contained in the vaccines. Thus, the ability to predict epidemic strains is inherently important in the use of these inactivated vaccines. In contrast, the vaccines based on live attenuated viruses may offer some cross-protection against circulating influenza strains that are not perfectly matched to the Flu strains in the vaccine [84]. These vaccines, administrated intranasally, contain live viruses that are attenuated (weakened) to not cause Flu illness and adapted to grow at 25°C by passaging at progressively lower temperatures to achieve cold adaptation and temperature sensitivity. Therefore, LAIV viruses replicate at the cooler temperatures found in the mucosa of the nasopharynx, but cannot infect the lower airways or lungs where warmer temperatures exist. They induce local mucosal neutralizing immunity and cell-mediated responses that may be longer lasting and more cross-protective than those elicited by chemically inactivated (killed) vaccine preparations [20]. Although they are very effective and immunogenic, live attenuated vaccines carry some risk and are potentially dangerous to immunosuppressed or immunodeficient individuals. Furthermore, the live attenuated viruses are highly genetically unstable. The obvious danger is that they could mutate and reassort with other Flu strains to generate new pandemic.

There are many requirements to obtain an effective vaccination. A vaccine must be safe, able to produce protective immunity in a very high proportion of the people to whom it is given and it must generate long-lived immunological memory. This means that both B and T cells must be primed by the vaccine. Finally, vaccines must be also cheap to manufacture if they are administered to large populations.
All registered influenza virus vaccines are produced using embryonated chicken eggs and the dose of vaccine is based on the HA content independently of the type of vaccine that is being used (the vaccine usually contains 15 μg of HA for each of the three strains; this dose is approximately the amount of purified virus obtained from the allantoic fluid of 1 infected embryonated egg). Before the beginning of the influenza season the vaccines are administered by intramuscular injection. For children who have never been vaccinated against influenza or who have never been exposed to the virus, it is recommended to vaccinate twice with a four-week interval. The vaccines can provide protection against disease in 70–90% of healthy adults [85]. The efficacy is lower (30–70%) in elderly patients, but protection against complication and mortality is achieved in 70–90% of elderly vaccinated [83].

1.3.2 Need to improve subunit influenza vaccine

Despite the fact that inactivated influenza vaccines are effective in preventing and curbing the spread of disease, there is room for new developments. Among the critical issues in developing new and better vaccines are the following: price per dose, speed of production, ease of production, choice of substrates to grow the virus in or to express viral antigens, cross-protection for variant strains, efficacy in general and in immunologically naive populations, safety, and acceptance by the regulatory agencies and the public [86].

A significant and recent development in the area of influenza vaccine research relies on the ability to genetically modify influenza viruses in a well-defined way. The technology which allows the artificial generation of influenza viruses is known as reverse genetics. The influenza viruses contain negative segmented genomes. The reverse genetics system is based on the intracellular transcription of wild-type or mutant viral RNAs from cDNAs inserted between polymerase promoters and terminator sequences in engineered plasmids.

Plasmids encoding both the viral RNA segments and the proteins needed to initiate the viral life cycle are transfected into eukaryotic cells for the rescue of infectious influenza virus [87]. This approach has proven to be extremely efficient, robust, and versatile, and has resulted in significant advances in both basic research and vaccine development.
Since the influenza virus possesses a segmented RNA genome, simultaneous infection of eggs with two different viruses may result in reassortment of segments to generate a new strain. Classically, the vaccine seed strains are produced by double infection of embryonated chicken eggs, using the recommended virus strain and the laboratory strain PR8 (which grows to high titers in these eggs), in order to produce a high growth reassortment. The use of reverse genetics offers several advantages over the classical reassortment approach: it is a more rational and direct approach compared with the potentially hit-or-miss traditional approach and it solves the problem of the possible presence of advantageous viruses in the epidemic virus isolate that could eventually contaminate the vaccine seed strain [88]. Finally, it permits the manipulation of viruses, including highly pathogenic subtypes, by introducing at the plasmid stage gene mutations, insertions or deletions to attenuate and optimize vaccine seed virus [89].

A further milestone is the licensure in 2007 by European regulators of the first seasonal influenza vaccine produced in mammalian cell line (Madin-Canin-Darby-Kidney, MDCK and Vero cells) rather than in eggs. The production of influenza vaccine in cell culture-based systems (FCC) offers significant advantages in the manufacturing process over egg-based production, particularly in relation to the imminent threat posed by pandemic strains. The production of influenza vaccines from viruses that have been propagated in embryonated chicken eggs presents some limitations: eggs must be ordered up to a year in advance, necessitating careful planning every year to ensure a sufficient supply of fertile eggs [90] and egg-based vaccine production cannot be scaled up at short notice to meet changes in demand or in serotype selection. Cell culture-based technology significantly reduces lead times and provides greater flexibility and viability of production because of the elimination of reliance on egg availability [91-95]. Cell stocks can be stored frozen and large quantities of vaccine can be generated on short notice [93 and 96]. This would be particularly advantageous in a pandemic situation, when demand for vaccine would increase with little or no warning. The virus also remains antigenically unchanged in cell lines, whereas it may become slightly modified during egg incubation [97]. In addition, influenza vaccines produced with cell cultures are believed to be safer than ones produced

in eggs and should not induce the hypersensitivity to egg-based vaccines experienced by children and adults, caused by the presence of some egg proteins in these vaccines [91].

An effective vaccine must induce the production of a good antibody titre. It is indeed the generation of neutralizing antibodies (IgA and IgG) that is thought to be responsible for the elimination of the virus following acute infection or protection following vaccination [98]. Most current influenza vaccines have been developed to induce neutralizing antibodies to the viral surface protein HA and also to the NA protein, which protect against viral infection by neutralizing virions or blocking the virus entry into cells. Unlike natural infections, intramuscular administration of inactivated vaccine, although eliciting serum antibodies (systemic IgG), does not usually induce mucosal immune responses (IgA), which are considered important for protecting the upper respiratory tract [99]. Therefore, an ideal influenza vaccine should induce IgA antibodies in the respiratory tract. Introduction of antigen via a non-mucosal route has been shown to be ineffective in turning on the mucosal immune system because of its compartmentalization. Therefore, there is a need for new vaccines and vaccination technologies that can effectively induce both systemic and mucosal immunity.

The humoral immune responses generated by current vaccine strategies target external viral coat proteins that are conserved for a given strain. Because of the high degree of antigenic drift among circulating influenza strains over the course of a year, antibody-mediated protection is effective against homologous viral strains but inadequate against heterologous strains with serologically distinct coat proteins. In addition, current subunit influenza vaccines are not effective at inducing cellular immune responses (particularly CTLs), directed at conserved viral epitopes, which may provide cross-protection against heterologous viral strains. A vaccine approach based on cell-mediated immunity may overcome this drawback. While nobody is suggesting abandoning the current antibody-based strategy, there is an increasing interest in the possibility that it might be useful to add a CD8+ T cell-activating component to the trivalent seasonal influenza vaccines [100]. Adoptive T cell transfer or cross-priming experiments with H1N1 A and H3N2 A viruses in mice have demonstrated that established CD8+ T cell memory is protective [101]. Primed mice may still show substantial weight loss and morbidity, but they clear serologically different influenza viruses from the lung more rapidly than mice that are not primed [101]. Cell-mediated responses typically focus on peptides from internal influenza

proteins, which are far less susceptible to antigenic variation and thus common to heterologous viral strains. It has been found that cross-reactive influenza-specific CTLs recognize peptides loaded on MHC class I molecules from the conserved internal influenza proteins, mainly the nucleoprotein (NP) and the structural matrix protein 1 (M1) [102-105]. This property gives vaccines that induce protective cellular immune responses the potential to protect against heterologous viral strains [106].
Several strategies have been proposed to address the need for vaccine able to confer a broader immune response, including the use of more conserved antigens such as conserved HA epitopes or M2 protein, the use of DNA encoding the whole NP and M1 proteins [107], administration of high dose vaccine or alternative modes of delivery such as intradermal or mucosal administration [108-110]. Although these approaches are promising, more research is necessary for the development of cross-protective vaccine and for the evaluation of the ability to induce a cross-reactive immune response [111].

Current subunit vaccines are designed to include only the antigens required for protective immunization. For this reason they offer the best safety profile in all age groups. However, the purity of the subunit antigens and the absence of the self-adjuvanting immunomodulatory components associated with attenuated or killed vaccines often result in a weaker immunogenicity. Formulation of vaccines with potent adjuvants is an attractive approach for improving the performance of vaccines composed of subunit antigens. Adjuvants have different mechanisms of action that will be examined in more detail in the following chapter.

1.4 Adjuvants

Unlike traditional vaccines based on attenuated live organisms, new subunit vaccines consisting of purified antigens are usually poorly immunogenic on their own and, thus, require the addition of compounds that can increase and modulate their intrinsic immunogenicity; these substances are called adjuvants.
Adjuvants can be used to improve the effectiveness of vaccine antigens in several different ways, including: 1) accelerating the generation of robust immune responses; 2) sustaining responses for a longer duration; 3) inducing local mucosal immune responses; 4) generating antibodies with increased avidity and neutralizing capability; 5) eliciting

cytotoxic T lymphocytes (CTLs); 6) enhancing immune responses in individual with weakened immune systems (for example children, elderly or immunocompromised individuals); 7) increasing the response rate in low-responder individuals; and 8) reducing the amount of antigen or the frequency of immunization required to provide protective immunity, thus reducing the cost of vaccination programs [112].

Given the new understanding of innate immune mechanisms, whose stimulation is now known to have an important role in the evolution of the adaptive immune response [113], adjuvants should be divided into two classes (delivery systems and immune-potentiators) based on their dominant mechanisms of action. Immune-potentiators activate innate immunity directly (for example, cytokines) or through PRRs (such as bacterial components) providing the pro-inflammatory context for antigen recognition, whereas delivery systems may concentrate and display antigens in repetitive patterns, target vaccine antigens to APCs, help colocalize antigens and immune-potentiators [114], protect the vaccine antigens from degradation and provide depot effects for vaccine antigens. Thus, both immune-potentiators and delivery systems can serve to increase antigen-specific immune response *in vivo*. Therefore, for subunit vaccines, the combination of delivery systems, immune-potentiators and the antigens (against which the adaptive immune responses are elicited) will be required to induce optimal immune responses (**Fig. 10**) [112].

Different classes of compounds display adjuvant activity in pre-clinical models; among them microbial products, mineral salts, emulsions, microparticles, nucleic acids, small molecules, saponins and liposomes, which exert their function by diverse and often poorly characterized mechanisms of action [112, 115 and 116]. However, only a few of them have been licensed for human use, while the vast majority failed due to an unacceptable safety profile [117].

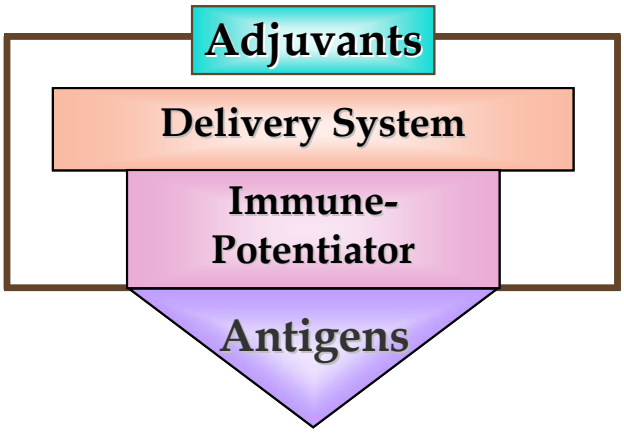


Fig. 10: Optimal vaccine formulation. Figure adapted from: Nature Reviews Drug Discovery 2, 727-735 (September 2003). Recent advances in the discovery and delivery of vaccine adjuvants. Derek T. O'Hagan & Nicholas M. Valiante.

The immune-potentiators act directly on immune cells that express the receptors for them, predominantly on DCs, inducing the up-regulation of cytokines, MHC class II and co-stimulatory molecules, and promoting DC migration to the T cell area of the lymph nodes [113 and 116]. One example of an immune-potentiator is represented by unmethylated **CpG** oligonucleotides (CpG) used as vaccine adjuvants in both preclinical and clinical studies [118 and 119]. CpG activate the innate immune response directly through TLR9, expressed by human plasmacytoid DCs (pDCs) and B cells [120], triggering the release of inflammatory cytokines [121] and biasing responses towards Th1 immunity and induction of CTL [122]. They act on B cells by stimulating their proliferation and IgM secretion [123]. In addition, CpG-activated B cells express increased levels of the Fcγ receptor and costimulatory molecules such as MHC class II, CD80, and CD86 [124-126]. In mature peripheral B cells, low concentrations of CpG DNA strongly synergize with signals through the BCR, leading to an approximate tenfold increase in B cell proliferation, antigen-specific Ig secretion and IL-6 secretion [124]. pDC have been shown to be directly activated by CpG DNA, which induces them to have growth factor–independent survival in culture, resistance to IL-4–induced apoptosis, increased surface expression of MHC class II, ICAM-1 and the costimulatory molecules CD40, CD54, CD80, and CD86, cytokine (IL-6, TNFα) and chemokine secretion, IFNα secretion, and maturation to become CD83 bright with increased activation of allogeneic T cells [127-130].

Three classes of CpG oligonucleotide ligands have been described by different sequence motifs and different abilities to stimulate IFNα production and maturation of pDCs. CpG-

A localizes to early endosomes, CpG-B localizes in late endosomes and CpG-C seems present in both types of endosomes [131]. Interestingly, the response of human pDCs is dependent upon the class of CpG ODN used to stimulate them. Stimulation with CpG-A induces sustained high IFNα production by pDCs but minimal upregulation of cell surface maturation markers CD80, CD86, and MHC class II [132-135] and has no effect on B cells. On the other hand, stimulation with CpG-B, a strong B-cell activator, results in increased expression of costimulatory and antigen-presenting molecules and enhances IL-8 and TNFα secretion but low levels of IFNα production by pDCs. Recently, a new class of CpG ODN, CpG-C, in which structural elements of CpG-A and CpG-B have been combined, has emerged, and this sequence activates B cells and induces IFNα production by pDCs [136].

The ability of CpG to induce potent Th1 immune responses, characterized by T cells secreting IFNγ and TNFα, is in marked contrast to the Th2 response induced by the traditional adjuvant, Alum, and is likely to overcome Th1-deficits in the immune response of the elderly [137]. The Th1 adjuvant effect of CpG appears to be maximized by its conjugation to protein antigens [121] or its formulation with delivery systems [138].

IC31 is a novel fully synthetic bi-component adjuvant which combines a novel immunostimulatory oligodeoxynucleotide containing deoxy-Inosine/deoxy-Cytosine (ODN1a) and the antimicrobial peptide KLKL5KLK (KLK). The ODN does not contain CpG motifs. It represents a promising novel adjuvant signaling via the TLR9 [139]. Apart from effective vaccine depot formation mediated by KLK [140], IC31 stimulates the activation of APCs and strongly induces antigen-specific cellular and humoral immune responses with type 1 dominance when combined with different types of antigens [141]. Interestingly, CTLs are induced, able to kill efficiently target cells in vivo. By contrast, adjuvants currently available for application in man, like Alum and MF59, predominantly induce type 2 responses and do not induce CTLs [142]. Importantly, IC31 does not have or induce systemic toxic effects and is locally very well tolerated [139]. In addition, systemic production of pro-inflammatory cytokines is not detectable upon IC31 administration, in contrast to CpG motifs, which are described to induce systemic side effects [143 and 144]. Therefore, IC31 seems to possess the requirements as a Flu vaccine adjuvant for application in humans, where strong antigen-specific cellular and humoral immune responses without induction of undesirable side effects are required.

While the cell wall lipopolysaccharide (LPS) of gram-negative bacteria is a potent TLR4 agonist, the toxicity profile of the natural product precludes its use in humans. Molecules mimicking lipid A, the domain of LPS molecule responsible for many of its immune activating abilities, have been widely reported. Such molecules, for example monophosphoryl lipid A (**MPL**), are effective vaccine adjuvants in animal models and humans, with suppressed toxicity while maintaining the ability to bind to TLR4 [145-147]. Importantly, for use in humans, MPL is always combined with delivery systems like alum or others [116 and 148]. Recently, a novel synthetic TLR4 agonist was developed, **E6020**, which is chemically well defined and has a promising safety profile based on investigations with animal models [149]. Structurally, E6020 consists of a simple hexa-acylated acyclic backbone, which allows for a more direct preparation of high-purity material than other synthetic TLR4 agonists [150 and 151]. Engagement of TLR4 promotes cytokine expression, antigen presentation and migration of APCs to the T cell area of draining lymph nodes, allowing for an efficient priming of naïve T cells.

The only adjuvants currently approved by the US Food and Drug Administration were incorporated in several human vaccines over 70 years ago in the form of insoluble aluminium salts (generically called Alum) [152]. **Alum** has a good safety record, its adjuvanticity is associated with enhanced antibody responses, but it induces a Th2, rather than a Th1 response [153]. In addition, Alum is not effective for the induction of mucosal IgA antibody responses. Although it has been used as an adjuvant for many years, its mechanism of action remains poorly defined. It has been proposed that alum acts through the formation of a depot because the adsorption to alum increases antigen availability at injection site inducing a gradual release and allowing an efficient uptake by APCs [154]. Alum could also increase the antigen uptake by DCs *in vitro*, further supporting an antigen delivery function [155]. However, several studies suggest that, in addition to antigen delivery, alum might have immunostimulating activities *in vivo*. Alum intramuscular administration results in the generation of a local inflammatory environment at the injection site characterized by the recruitment of blood cells, including inflammatory monocytes [116 and 156]. In addition, it has been recently shown that alum is capable of activating caspase-1 and inducing the release of the potent pro-inflammatory cytokines IL-1β, IL-18 and IL-33 [157]. This adjuvant effect has been demonstrated to be mediated by the activation of Nalp3, a member of an intracellular PRR, which along with the adaptor

molecule ASC is a key component of the multiprotein complex termed inflammasome [158].

In other countries, including members of the EU, other vaccine adjuvants have been approved for human use. The **MF59** adjuvant, a squalene-based oil-in-water emulsion, has been included in a licensed influenza vaccine (Fluad) for more than a decade, therefore a significant amount of clinical data describing its potency and safety are available [159]. It has been shown to be a potent stimulator of cellular and humoral responses to subunit antigens in both animal models and clinical studies [160]. MF59-adjuvanted vaccine showed significantly increased antibody titers and enhanced cross-reactivity compared to non-adjuvanted vaccine formulations [161]. MF59 was also evaluated as an adjuvant for a potential pandemic vaccine and induced significantly higher antigen-specific antibody responses and superior cross-neutralization in human subjects [162 and 163]. Importantly, MF59 also allowed a significant reduction in the antigen dose, while maintaining the potency of the vaccine, a finding that might be important to allow an increase in the number of people immunized when an influenza pandemic occurs, assuming vaccine is available [162]. Despite the fact that MF59 is widely used as a flu vaccine adjuvant, its mechanism of action is only partially understood. Similarly to alum, MF59 could promote antigen uptake by APCs *in vivo* [164]. Besides promoting antigen delivery, MF59 might also act as a local pro-inflammatory adjuvant, by inducing an infiltration of blood mononuclear cells at the injection site [165]. Hence, it appears that, following immunization, MF59 enhances the immune response at a range of points, including the induction of chemokines to increase recruitment of immune cells to the injection site, enhanced antigen uptake by monocytes at the injection site and enhanced differentiation of monocytes into DCs, which represent the gold-standard cell type for priming naive T cells. A particularly important feature of MF59 may be that it strongly induces the homing receptor CCR7 on maturing DCs, thus facilitating their migration into draining lymph nodes where they can trigger the adaptive immune response specific to the vaccine [159].
MF59 is particularly effective at enhancing antibody and T-cell proliferative responses and it is generally a more potent adjuvant than Alum and CpG [137]. Nevertheless, it is not a potent adjuvant for the induction of Th1 cellular immune responses in preclinical models [166] and more potent Th1 responses may be required to provide protective immunity against influenza viruses. Th1 immune-potentiators, including CpG oligonucleotides, have been added to MF59, both to improve potency and to alter the kind

of response induced. MF59 induces greater IgG titres than CpG alone while the combination of both provides significantly greater titers than either CpG or MF59 [137]. CpG induces Th1 cytokines and antibodies of the IgG2a isotype, while MF59 promotes a strong Th2 immune response, associated with high levels of the IgG1 antibody isotype [167]. However, it has been demonstrated that the adjuvanticity of MF59 is modulated by the addition of CpG, which induces a dramatic shift from a Th2 to a Th1 response in BALB/c mice [137].

Hence, it appears possible to increase the efficacy of MF59 adjuvant by adding an additional immune potentiator, such as a TLR-dependent adjuvant. The challenge for vaccine developers is to determine how best to use combination adjuvants, to achieve optimal immune activation and yet avoid unwanted toxic reactions.

1.5 Aim of the project

Influenza is still a significant cause of morbidity and mortality, with seasonal epidemics occurring worldwide. Therefore, improved vaccines that induce a broader and more potent immune response are needed to provide protection to the populations most at risk.

Formulation of vaccines with potent adjuvants is an attractive approach for enhancing the performance of vaccines composed of subunit antigens and offers the opportunity to drive the immune response into a desired Th profile.

In the present study we evaluated different adjuvants (MF59, CpG, E6020 and IC31) alone and in combinations for their ability to enhance and modulate antibody and T cell responses induced by subunit influenza vaccines in mice.

Although the main effector mechanisms in the viral clearance appear to be the antiviral antibodies and the killing of virus-infected cells by CD8+ T lymphocytes, CD4+ T cells are also important in regulating immune responses to infection: antigen-specific CD4+ T cells provide cognate help to B cells, a requisite event for immunoglobulin switch and affinity maturation of B cells that produce neutralizing antibodies; they provide help to cytotoxic CD8+ T cells, critical for their expansion and persistence as memory cells; finally, CD4+ T cells may participate directly in viral clearance via cell-mediated cytotoxicity or through production of cytokines. Hence, to assess the immunogenicity and compare the efficacy of the different Flu vaccine formulations, Ag-specific CD4+ T cells were analyzed for cytokine production by intracellular cytokine staining (ICS) after *in vitro* restimulation of splenocytes from immunized mice; in addition, humoral immune responses were analyzed by measuring HA-specific serum antibody titres (total IgG and IgG1 and IgG2a isotypes) by ELISA.

In appropriate mouse strains and in SPF (specific pathogen free) conditions, subunit influenza vaccine promotes a type-2 immune response. MF59 adjuvant enhances this type-2 response by increasing both IgG1 antibody and Th2 CD4+ T cell responses, but it is poor for the shift of this response towards a Th1 response, which may be important for the resolution of influenza infections. Since the quality of the immune response can be modulated by the selection of appropriate types of adjuvants or their combinations, we analyzed the protective capacities of a Th2 vs. a Th1 promoting Flu vaccine and compared the different types of immunity induced by vaccination with that induced by influenza virus infection. For this purpose and to determine which vaccine formulation is more efficient at inducing protection against infection, we also determined the neutralizing

antibody titres induced after two immunizations and the survival of mice after lethal challenge with influenza virus. In addition, to separately investigate the contribution of humoral components in protection against infection, sera from mice differently immunized were collected and transfer into naïve mice subsequently challenged with homologous influenza virus.

Finally, the immune responses induced by different vaccine formulations in naïve mice were compared with those induced in mice previously infected with influenza virus. The latter situation is more similar to that found in humans, who are often reinfected annually with the prevalent circulating influenza strains, and it is important to see if this condition influences the outcome of vaccination.

Our investigations have significant implications for the development of new and improved Flu vaccines against pandemic and inter-pandemic influenza virus strains. They offer the opportunity to establish which type of immune response is more effective in the protection against viral infection and open the possibility to drive it into a desired direction by choosing appropriate adjuvants or combinations thereof.

2. SUMMARY

2.1 English Version

Seasonal epidemics and the threat of a pandemic of influenza virus are a significant burden to public health, and therefore, improved vaccines that induce a broader and more potent immune response are needed to provide better protection. Formulation of vaccines with potent adjuvants is an attractive approach for enhancing the performance of vaccines composed of subunit antigens. In the present study we evaluated different adjuvants (MF59, CpG, E6020 and IC31) alone and in combinations for their ability to enhance and modulate antibody and CD4+ T cell responses induced by subunit influenza vaccines. As individual adjuvant, MF59 induces optimal cellular and humoral responses, whereas none of the tested immune-potentiators (CpG, E6020 or IC31) when administered as single adjuvant with the influenza vaccine are able to induce as high CD4+ T cell or antibody responses as MF59 does. On the other hand, MF59 is not able to induce effective Th1 responses, which could be achieved by the addition of an immune-potentiator. The addition of CpG to MF59 allows the induction of a potent Th1 response; also the combination of MF59 with E6020 and IC31 immune-potentiators results in a shift of the immune responses from a Th2- toward a more pronounced Th1-type, but in these cases the shift is less marked, maintaining a more balanced immunity. Thus, choosing the appropriate adjuvant combinations opens the possibility to drive and modulate the quality of the immune response into a desired direction.

Further investigations on the protective capacity of a Th2 vs. a Th1 promoting Flu vaccine (adjuvanted respectively with MF59 adjuvant and MF59 + CpG) demonstrated that both types of adjuvanted vaccines induce levels of neutralizing antibody titres higher than those generated by exposure to sublethal doses of influenza virus, and both provide protection from lethal challenge with homologous influenza virus. However, pre-exposed mice exhibit minor weight loss than mice immunized with adjuvanted vaccines when challenged with a lethal dose of virus.

Finally, the immune responses induced by different vaccine formulations in naïve mice were compared with those induced in mice previously infected with influenza virus, a situation more similar to that found in humans. In contrast to naïve mice, mice pre-exposed to influenza virus showed a clear Th1 response to MF59, suggesting that the pre-established immune status influences the outcome of vaccination.
Our investigations have significant implications for the development of new and improved Flu vaccines against pandemic and inter-pandemic influenza virus strains. They offer the opportunity to establish which type of immune response is more effective in the protection against viral infection and open the possibility to drive it into a desired direction by choosing appropriate combinations of adjuvants.

2.2 Italian Version

Le epidemie stagionali e la minaccia di una pandemia causata dal virus dell'influenza sono un onere significativo per la salute pubblica, quindi vaccini potenziati in modo da indurre una più ampia e più potente risposta immunitaria sono necessari per garantire una migliore protezione. La formulazione dei vaccini con potenti adiuvanti è un approccio interessante per aumentare le prestazioni dei vaccini composti da antigeni di superficie purificati. In questo studio abbiamo valutato diversi adiuvanti (MF59, CpG, E6020 e IC31) somministrati da soli o in diverse combinazioni per la loro capacità di migliorare e modulare le risposte anticorpali e dei linfociti T CD4+ indotte dai vaccini antinfluenzali. Come singolo adiuvante, MF59 promuove ottime risposte cellulari e umorali, mentre nessuno degli immunopotenziatori testati (CpG, E6020 o IC31), quando somministrato come unico adiuvante con il vaccino antinfluenzale, è in grado di indurre elevate risposte cellulari o anticorpali come MF59. D'altra parte MF59 non è in grado di promuovere un'efficace risposta Th1, che potrebbe essere ottenuta con la contemporanea somministrazione di un immunopotenziatore. L'aggiunta di CpG a MF59 permette l'induzione di una potente risposta Th1; anche la combinazione di MF59 con E6020 e IC31 comporta il cambiamento del profilo della risposta immunitaria da un tipo Th2 verso uno più marcatamente Th1, ma in questi casi il passaggio è meno netto e viene mantenuta una risposta qualitativamente più bilanciata. Quindi, la scelta delle opportune combinazioni di adiuvanti offre la possibilità di modulare e indirizzare la qualità della risposta immunitaria verso una determinata direzione.

Ulteriori indagini sulla capacità protettiva di un vaccino antinfluenzale che promuove una risposta Th2 rispetto ad uno che ne promuove una di tipo Th1 (adiuvati, rispettivamente, con l'adiuvante MF59 e con MF59 + CpG) hanno dimostrato che entrambi i tipi di vaccino inducono titoli di anticorpi neutralizzanti superiori a quelli generati a seguito dell'esposizione a dosi subletali di virus dell'influenza; entrambi, inoltre, conferiscono protezione nei confronti del challenge con una dose letale del virus dell'influenza. Tuttavia, dopo il challenge i topi pre-esposti al virus manifestano una minor perdita di peso rispetto ai topi immunizzati con vaccini adiuvati.

Infine, le risposte immunitarie indotte dalle diverse formulazioni vacciniche in topi naive sono state confrontate con quelle indotte in topi precedentemente infettati con il virus dell'influenza, una situazione questa che meglio rispecchia quella riscontrata negli esseri umani. A differenza dei topi naive, i topi pre-esposti al virus mostrano una netta risposta Th1 dopo la somministrazione del vaccino con MF59, suggerendo che lo "stato immunitario" pre-esistente influenzi il risultato della vaccinazione.

Le nostre indagini hanno implicazioni significative per lo sviluppo di nuovi e migliori vaccini contro l'influenza pandemica ed inter-pandemica. Questi studi offrono la possibilità di stabilire quale tipo di risposta immunitaria sia più efficace nella protezione contro le infezioni virali e la possibilità di dirigerla verso una determinata direzione, attraverso la scelta delle opportune combinazioni di adiuvanti.

3. MATERIALS & METHODS

3.1 Materials

3.1.1 Mice

Pathogen-free female BALB/c and C57BL/6 mice 6-8 weeks of age were purchased from Charles Rivers Laboratories. All animals were housed and treated according to internal animal ethical committee and institutional guidelines.

3.1.2 Vaccines

Trivalent influenza vaccines composed of equal amounts of haemagglutinin (HA) from an influenza A H1N1 subtype virus, an influenza A H3N2 subtype virus, and an influenza B virus (Novartis Vaccines, Siena, Italy) or monovalent H1N1 vaccine containing a single antigen derived from influenza strain H1N1 A/Solomon Islands/3/2006 (Novartis Vaccines, Siena, Italy) were used in the experiments for mouse immunization. The trivalent and monovalent vaccines contain purified subunit antigens and are standardized for HA content by single-radial-immunodiffusion as recommended by regulatory authorities.

Subunit influenza vaccines are produced from virus grown in the allantoic cavity of embryonated chicken eggs inoculated with a specific type of influenza virus suspension. Each strain is grown separately. Then the allantoic virus is purified, concentrated and chemically inactivated. The reactogenicity associated with purified influenza virus is greatly reduced by the treatment with a detergent. Additionally, to produce subunit

vaccines the surface glycoprotein antigens are isolated and purified. On average, between one and two eggs are needed to produce one dose of vaccine.

Virus strains included in trivalent vaccines were:

- H1N1 A/New Caledonia/20/1999, H3N2 A/Wyoming/3/2003 and B/Jiangsu/10/2003 (trivalent Flu vaccine for the 2004-2005 season);
- H1N1 A/New Caledonia/20/1999, H3N2 A/New York/55/2004 and B/Jiangsu/10/2003 (trivalent Flu vaccine for the 2005-2006 season);
- H1N1 A/Solomon Islands/3/2006, H3N2 A/Wisconsin/67/2005 and B/Malaysia /2506/2004 (trivalent Flu vaccine for the 2007-2008 season).

3.1.3 Virus

H1N1 A/Solomon Islands/3/2006 influenza virus was grown in Madin-Darby canine kidney (MDCK) cells.

MDCK cells were maintained as a monolayer in DMEM (Dulbecco's modified Eagle's medium, Gibco) containing 10% FCS (fetal calf serum, Hyclone) and PSG (penicillin-streptomycin-L-glutamine, Gibco) at 37°C, in a humidified atmosphere of 5% CO_2, and they were periodically passaged as soon as they reach 80-90% of confluence.

To expand the virus, MDCK cells were grown to 80% confluence (about $7x10^6$) on 75 cm^2 flasks. At the moment of infection, growth medium was replaced with MEM (Minimum Essential Medium, Gibco) containing PSG and TPCK (tosylsulfonyl phenylalanyl chloromethyl ketone)-treated trypsin (1:4000) plus the virus at multiplicity of infection (MOI) of 10^{-4}. At 24, 48 and 72 hours the supernatant was collected and clarified of cellular debris by low speed centrifugation. The virus was then concentrated by ultracentrifugation (Beckman centrifuge, SW40 tubes) at 28.000 rpm for 4 h at 4°C through a cushion of 20%(w/v) sucrose solution prepared in TNE (0,02 M Tris-HCl pH8, 150 mM NaCl, 2 mM EDTA). The pellet was resuspended in 0,5 ml of PBS (Phosphate buffered saline) and titrated.

Virus titration: $1,5x10^4$ MDCK cells/well were seeded in 96-well flat plates in MEM/3% FCS/PSG 24 h before the titration.

The medium was replaced with MEM/PSG/ TPCK-trypsin (1:4000) and viral suspension stock was inoculated for eight replicas, to be passaged in 10-fold serial dilutions starting

from an initial dilution of 1:10. Cells were grown at 37°C, 5% CO_2. After 72 h, the number of wells in which MDCK cells were lysed were evaluated at the microscope and the $TCID_{50}$ (50% tissue culture infectious dose) was calculated according to Spearman-Kärber method [168].

3.1.4 Adjuvants

MF59

MF59 emulsion was obtained from Novartis Vaccines, Marburg, Germany. The oil in water emulsion MF59, consisting of 4.3% squalene, 0.5% Tween 80, 0.5% Span 85 (Sigma, St. Louis, MO) in citrate buffer, was manufactured as previously described [169]. In brief the emulsion was prepared by homogenization at 12,000 psi with a microfluidizer (Microfluidics, Newton, MA). The mean particle size of the emulsion droplets was determined with a Mastersizer X (Malvern Instruments, Southborough, MA). The emulsion was made sterile by passage through a polysulfone filter (220 nm pore size; Gelman Sciences, Ann Arbor, MI) and then stored at 4°C [170].

CpG

The CpG oligonucleotide (5′-TCC ATG ACG TTC CTG ACG TT-3′), previously described as 1826 was synthesized with a phosphorothioate backbone by Oligos Etc. (Wilsonville, OR), ethanol precipitated, and re-suspended in 10 mM Tris (pH 7.0) 1 mM EDTA for storage at −80°C.

E6020

The synthetically produced TLR4 agonist E6020, a structural mimic of lipid A (the simplest active forms of lipopolysaccharide), was obtained from the Eisai Research Institute (Andover, MA).

IC31

IC31 consisting of an antibacterial peptide (11-mer cationic peptide $KLKL_5KLK$) and a TLR9 agonist, a synthetic ODN1a, without a CpG motif, was produced by Intercell AG (Vienna, Austria). $KLKL_5KLK$ (NH_2-KLKLLLLLKLK-COOH) was manufactured by

Bachem AG, Switzerland, and ODN1a (phosphodiester backbone ODN, oligo-$(dIdC)_{13}$) was synthesized by Transgenomics, USA.

3.2 Individual vaccine adjuvant formulations

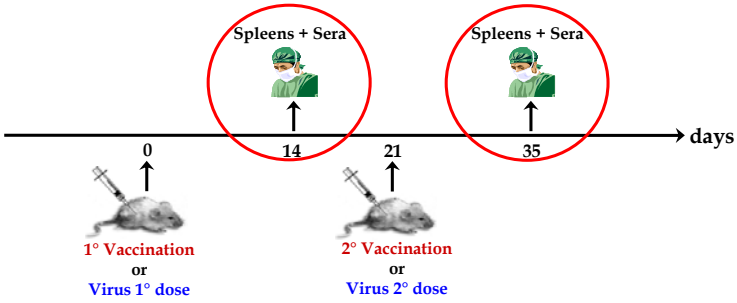
For MF59 adjuvanted vaccine formulations, influenza vaccine was prepared by mixing MF59 (*v*/*v*) 1:1 with either trivalent vaccine to a final concentration of 0.3 μg/dose of trivalent vaccine (0.1 μg each antigen) or monovalent vaccine at 0.2 μg/dose of vaccine antigen (derived from influenza strain H1N1 A/Solomon Islands/3/2006) and Phosphate Buffered Saline (D-PBS 1X, Gibco).
In the formulations containing CpG, E6020 or IC31, immune-potentiators were added to the formulations prior to immunizations by simple mixing with the trivalent or monovalent influenza vaccines at respective doses. CpG was added at 25, 10, 1 or 0.1 μg/dose, E6020 at 10 μg/dose and IC31 at high (IC31h = 100 nmol KLK/4 nmol ODN1a) or low (IC31l = 10 nmol KLK/0.4 nmol ODN1a) dose.

3.3 Preparation of MF59 emulsion containing immune-potentiators

In the formulation containing the immune-potentiator E6020 in MF59, E6020 was dissolved at 1 mg/ml in $CHCl_3$, and added to the squalene fraction. Before homogenization, $CHCl_3$ was completely evaporated from the squalene fraction. The final concentration of E6020 within the emulsion was 200 μg/ml, and the mean particle size of the emulsion was 162 ± 20 nm with a polydispersity index (PDI) of 0.11. E6020 formulated into MF59 was mixed with influenza vaccine at respective doses.
In the formulations containing MF59 plus either IC31 or CpG, immune-potentiators were mixed with the vaccine antigens at respective doses (see above) and MF59 was added (*v*/*v*) 1:1 immediately before immunizations.

3.4 Experimental design

3.4.1 Immunizations/infections



Groups of 8-10 BALB/c or C57BL/6 mice were used for experiments reviewed and approved by the institutional review committees. Animals were immunized two times at 3-week intervals in the tibialis anterior muscles in the two hind legs of each animal with 50 µl/leg (100 µl total per mouse). Doses were 0.3 µg/mouse (0.1 µg each antigen) for Balb/c mice and 0.3 or 0.9 µg/mouse (0.1 or 0.3 µg each antigen, respectively) for C57BL/6 mice of soluble trivalent influenza vaccine alone or mixed with: MF59 adjuvant; CpG oligonucleotides; E6020; IC31h; IC31l; MF59 + CpG; MF59 + E6020; MF59 + IC31h; and MF59 + IC31l.

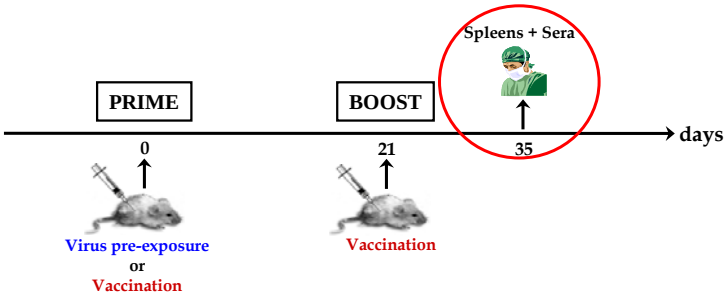
In some experiments the monovalent Flu vaccine (0.2 µg of Ag derived from influenza strain H1N1 A/Solomon Islands/3/2006) was used instead of the trivalent vaccine.

Alternatively, mice were intranasally infected with one or two sublethal doses (10^3 $TCID_{50}$) of influenza virus strain H1N1 A/Solomon Islands/3/2006 diluted in PBS.

Samples sera (of 3, 5 or 8 mice per group) and spleens (of three mice per group) were collected at 2 weeks following the 1st treatment, and at 2 weeks or 4 months following the 2nd treatment.

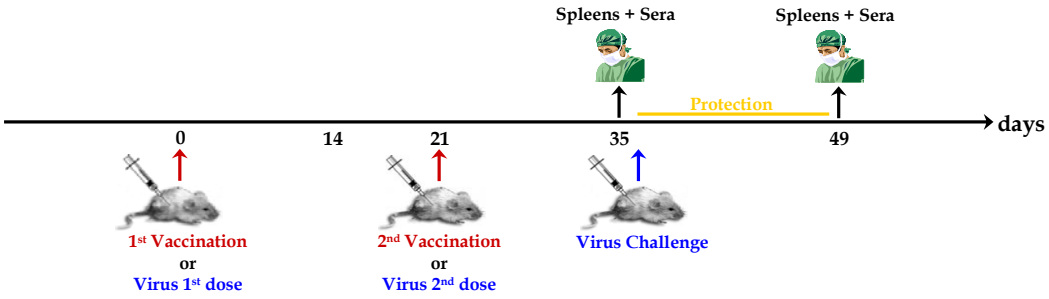
Immunogenicity was measured in serum samples determining total IgG (immunoglobulin G) antibody titres by ELISA. Neutralizing efficacy of serum antibodies was evaluated by a Microneutralization Assay. Th1/Th2 type responses were measured by titration of HA-specific IgG subclasses 1 and 2a in serum samples by ELISA and by monitoring the antigen-specific CD4+ T-cell cytokine responses in splenocytes.

3.4.2 Pre-exposure experiment



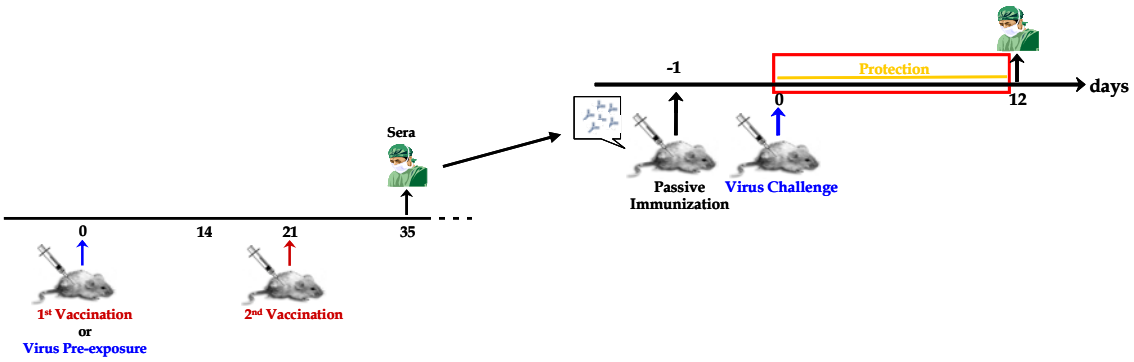
Balb/c mice were divided in three groups and differently primed: group 1 was treated with PBS; group 2 was pre-exposed to a sublethal dose of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon Islands/3/2006); and group 3 was immunized with monovalent Flu vaccine (0.2 µg of Ag derived from influenza strain H1N1 A/Solomon Islands/3/2006) + MF59. 3 weeks after these treatments each group was further divided in 3 subgroups and boosted with monovalent Flu vaccine alone, monovalent Flu vaccine + MF59 or PBS. At 2 weeks after boost mouse spleens and sera were taken and analyzed respectively for CD4+ T cell and antibody responses.

3.4.3 Challenge experiment



Two weeks after the final treatment, 6 mice per group were challenged intranasally with 30 µl of virus solution containing 10 mouse 50% lethal doses (MLD_{50}) of the H1N1 A/Solomon Islands/3/2006 influenza virus strain diluted in PBS. After challenge, mice were monitored daily for survival and weight loss for 14 days. At the end of the challenge study, mice that survived the challenge were killed and serum samples and spleens were collected for the analysis of antibody and T cell responses.

3.4.4 Passive immunization experiment



Balb/c mice were intranasally inoculated with a sublethal dose of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon Islands/3/2006) or immunized intramuscularly twice with the monovalent Flu vaccine (0.2 µg of Ag derived from influenza strain H1N1 A/Solomon Islands/3/2006) either alone or adjuvanted. Sera were collected 2 weeks after 2^{nd} immunization or 5 weeks after exposure to virus and pooled sera from mice vaccinated or infected were administrated intra-abdominally to naive mice. 24 hours later, mice were challenged intranasally with 3 MLD_{50} of H1N1 A/Solomon Islands/3/2006 influenza virus. In the control group, the virus used for challenge was pre-incubated with sera from mice pre-exposed to virus. Survival and average weight loss were followed for two weeks after challenge.

3.5 Serological assays

3.5.1 Detection of antigen-specific antibodies in mouse sera by enzyme-linked immunosorbent assay (ELISA)

Titration of HA-specific IgG (immunoglobulin G), total and subclasses 1 and 2a, and IgA was performed on individual or pooled sera 2 weeks after the last immunization/infection or 2 weeks after the lethal challenge. PolySorp 96-well plates for IgG total and subclasses detection and MaxiSorp 96-well plates for IgA detection (both Nunc, Roskilde, Denmark) were coated overnight at 27–30°C with 0.2 µg/well of H1N1 A, H3N2 A or B antigens in

PBS. Wells were washed with PBS containing 0.1% Tween 20 (wash buffer) and blocked for 1 h at room temperature with 300 µl of 3% poly vinyl pyrolidine. Wells were then washed and serum samples and serum standard, initially diluted 1:200–1: 2000 in PBS/1% BSA (bovine serum albumin)/0.05% Tween-20, were transferred into coated-blocked plates and serially diluted twofold (final volume of 100 µl/well). After 2h of incubation at 27-30°C, plates were washed and 100 µl/well of each secondary antibody were added and left for additional 2 h. Antigen-specific total IgG, IgG1, IgG2a and IgA antibodies were revealed with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co., SA Louis, Mo.), IgG1, IgG2a or IgA (all Southern Biotechnology Associates, Birmingham, AL), respectively. After three washes, 100 µl of the substrate solution, 10 mg of PNPP (p-nitrophenyl phosphate disodium salt) dissolved in 10 ml of Diethanolamine Substrate Buffer, were added to each well for approximately 20 min and the absorbance was measured at 450 nm with a Spectramax 340 spectrophotometer and analyzed with Softmax Pro software (Molecular Devices, Sunnyvale, CA).
Antibody titres are those dilutions that gave an optical density (OD) higher than the mean plus five times the standard deviation of the average OD obtained in the pre-immune sera. The titres were normalized with respect to the reference serum assayed in parallel. Geometric mean titres (GMT) 2 weeks after the first immunization (post-1) of eight mice per group, 2 weeks after the second immunization/infection (post-2) of three or five mice per group and 2 weeks after the lethal challenge of three mice per group were calculated. IgG2a:IgG1 titre ratios were calculated using the respective geometric mean titres.

3.5.2 Microneutralization assay

The microneutralization assay was performed with MDCK cells. $1.5x10^4$ MDCK cells/well were seeded in 96-well flat plates in MEM/3% FCS/PSG 24 h before neutralization assay.
Serum samples were heat inactivated for 30 min at 56°C and diluted 1:20 in MEM/PSG medium. This 1:20 starting dilution of inactivated sera was serially diluted three-fold in 50 µl of medium in 96-well bottom plates (two replicate wells for each dilution). The diluted sera were mixed with an equal volume (50 µl/well) of medium containing H1N1

A/Solomon Islands/3/2006 influenza virus at 300 $TCID_{50}$/50 µl/well and incubated for 1 h at 37°C in a 5% CO_2 humidified atmosphere.

During the incubation time the medium of the MDCK cells plated the day before was replaced with 100 µl/well of MEM/PSG/ TPCK-trypsin (1:2000). Subsequently, 100 µl of the virus and serum mixture were added to MDCK cells ($1.5x10^4$ per well).

Each plate contained the following controls: four wells of virus control solution containing the working dilution of virus, the cells and the medium (positive control); four wells of cell control solution containing only the cells and the medium (negative control). Each assay contained a back titration of the virus used.

The plates were incubated for 22 h at 37°C in a 5% CO_2 atmosphere. Then, cells were washed with PBS and fixed with 2% PFA (paraformaldeyde, Sigma-Aldrich) at room temperature for 15 min.

The presence of viral proteins was detected with a mouse anti-nucleoprotein and matrix monoclonal antibody conjugated to fluorescein isothiocyanate (FITC) specific for influenza A virus proteins (IMAGEN^{TM} Influenza Virus A and B kit, Dako, Glostrup, Denmark).

The ELISA was performed at room temperature. The fixed plates were washed three times with PBS containing 0.1% Tween 20 (wash buffer) and blocked with 100 ml/well of PBS/0.1% BSA/ 0.1% Tween 20 for 15 min. The plates were washed in wash buffer. The anti-M/NP primary antibody diluted 1:100 in PBS/0.1% BSA/0.1% Tween 20 was added to each well (30 µl/well). The plates were incubated at room temperature for 90 min with agitation, in the dark. Then, they were washed three times in wash buffer and the anti-FITC secondary antibody, conjugated with horseradish peroxidase (HRP) (Roche), was diluted 1:1000 in PBS/0.1% BSA/0.1% Tween 20 and added to each well (30 µl/well). The plates were incubated at room temperature for 90 min with agitation, in the dark and then washed six times with wash buffer. The substrate solution was freshly prepared by dissolving a 20 mg tablet of o-Phenylenediamine dihydrochloride (Sigma) in 40 ml of 0.05 M phosphate-citrate buffer, pH 5.0, and by adding 40 µl of 30% hydrogen peroxide (H_2O_2) immediately prior to use. 100 µl of substrate solution were added to each well, and the plates were incubated at room temperature for approximately 5 min in the dark. The absorbance was measured at 450 nm (A_{450}) with a Spectramax 340 spectrophotometer and analyzed with Softmax Pro software (Molecular Devices, Sunnyvale, CA).

Absorbance values correlated directly to the number of infected cells in the wells, and were used to calculate neutralizing antibody titers.

The average A_{450} was determined for quadruplicate wells of virus-infected and uninfected control wells and also for duplicate wells of each sample. The percentage of virus neutralization for each serum dilution was determined using the following equation:

$$= 100 - \left(\frac{\text{mean } A_{450} \text{ value of serum dilution - mean } A_{450} \text{ value of negative control}}{\text{mean } A_{450} \text{ value of positive control}} \text{ x } 100 \right)$$

The serum dilution that neutralized 80% of the virus (IC_{80}) was calculated by interpolation and expressed as the reciprocal dilution of the sample giving an 80 % reduction in viral antigen. IC_{80} values correlate with serum neutralizing antibody titres.

3.6 Antigen-specific CD4+ T-cell cytokine Response

Three mice per treatment were sacrificed, spleens were collected in ice-cold complete RPMI 1640 (Gibco) containing 2.5% FCS, β-mercaptoethanol (50 μM) and antibiotics and placed into 70 μm cell strainers (BD Falcon, Marsbourg, MA, USA) on top of 50 ml tubes. Spleens were meshed individually or in pools by using the back of a syringe plunger. Single cell suspensions were obtained. Red blood cells were lysed with the ACK lysis buffer (NH_4Cl 0.15 M, $KHCO_3$ 1 mM and EDTA 0.1 mM, pH 7.4) and splenocytes were resuspended in complete RPMI. Duplicate samples of splenocytes were cultured 200 μl/well at 2×10^6 cells per well in round-bottom 96-well plates in complete RPMI. Cultures were stimulated in the presence of anti-CD28 antibody (1 μg/ml) (Becton–Dickinson) plus either a mixture of the three influenza antigens (1 μg/ml each) or 3 μg/ml of a single vaccine antigen, with anti-CD28 antibody alone (unstimulated, <0.1% total cytokine-positive cells) as negative control, or with anti-CD28 antibody plus anti-CD3 antibody (1 μg/ml) (Becton–Dickinson) as positive control. After 4 h of stimulation, Brefeldin A (5 μg/ml) (Sigma-Aldrich) was added for additional 12 h. Cells from duplicate wells were pooled, washed and stained with LIVE/DEAD Aqua Fixable Dead Cell Stain (Invitrogen, Breda, The Netherlands) to exclude dead cells in the analysis. After staining, cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences Pharmingen), and stained with the following monoclonal antibodies: Pacific Blue-conjugated anti-CD4,

PerCP-Cy5.5-conjugated anti-CD3, FITC-conjugated anti-IFNγ, Alexa 700-conjugated anti-TNFα, PE-conjugated anti-IL-5 and APC-conjugated anti-IL-2 (all Becton–Dickinson). Cells were acquired on a LSR-II (Becton–Dickinson) and analyzed using FlowJo software (Tree Star). The percentage of CD4+ T cells producing cytokines is represented in multicolor bar graphs, with each bar showing the response of splenocytes from three pooled spleens or the mean response of the spleens of three individually analyzed mice. The percentages of the unstimulated samples were subtracted from the antigen-stimulated samples.

The ratios between the IFNγ and the IL-5 producing T cells were calculated using the respective percentages.

4. RESULTS

4.1 Evaluation of cellular and humoral immune responses to influenza vaccines

Both antibody and cell-mediated responses are involved in the immune response to influenza virus.

Vaccination induces an influenza virus-specific immune response which is generally documented as the generation of antibodies that react specifically with the virus and mediate protection against the infection. Also specific immune cells participate in defense against the virus, in particular CD4+ T helper cells and CD8+ cytotoxic T lymphocytes (CTL) which are part of the immune response induced by vaccination [171].

Although CD4+ T helper cells themselves may not have a primary role in the clearance of acute viral infections, they play a crucial role in the control and regulation of the humoral and cell-mediated immune response [172]. Several subsets of T helper cells are distinguished based on the cytokines they produce. T helper type 1 cells (Th1) preferentially produce IFNγ and IL-2, whereas Th2 preferentially produce IL-4 and IL-5. These cytokine responses contribute to the regulation of both the antibody and the CTL responses to vaccination. While both Th1 and Th2 provide T cell help for antibody production, an increased Th1 relative to the Th2 response to vaccination is needed for optimal stimulation of CTL responses [173]. CTL have a key role in clearing influenza virus, but generally they are poorly stimulated by current vaccines, that is why CD8+ T cell responses were not measured in our system.

Since CD4+ T cell help is also necessary for antibody responses, to evaluate the immunogenicity and compare the efficacy of different influenza (Flu) vaccine formulations, both CD4+ T cell and humoral immune responses were analyzed using a mouse model.

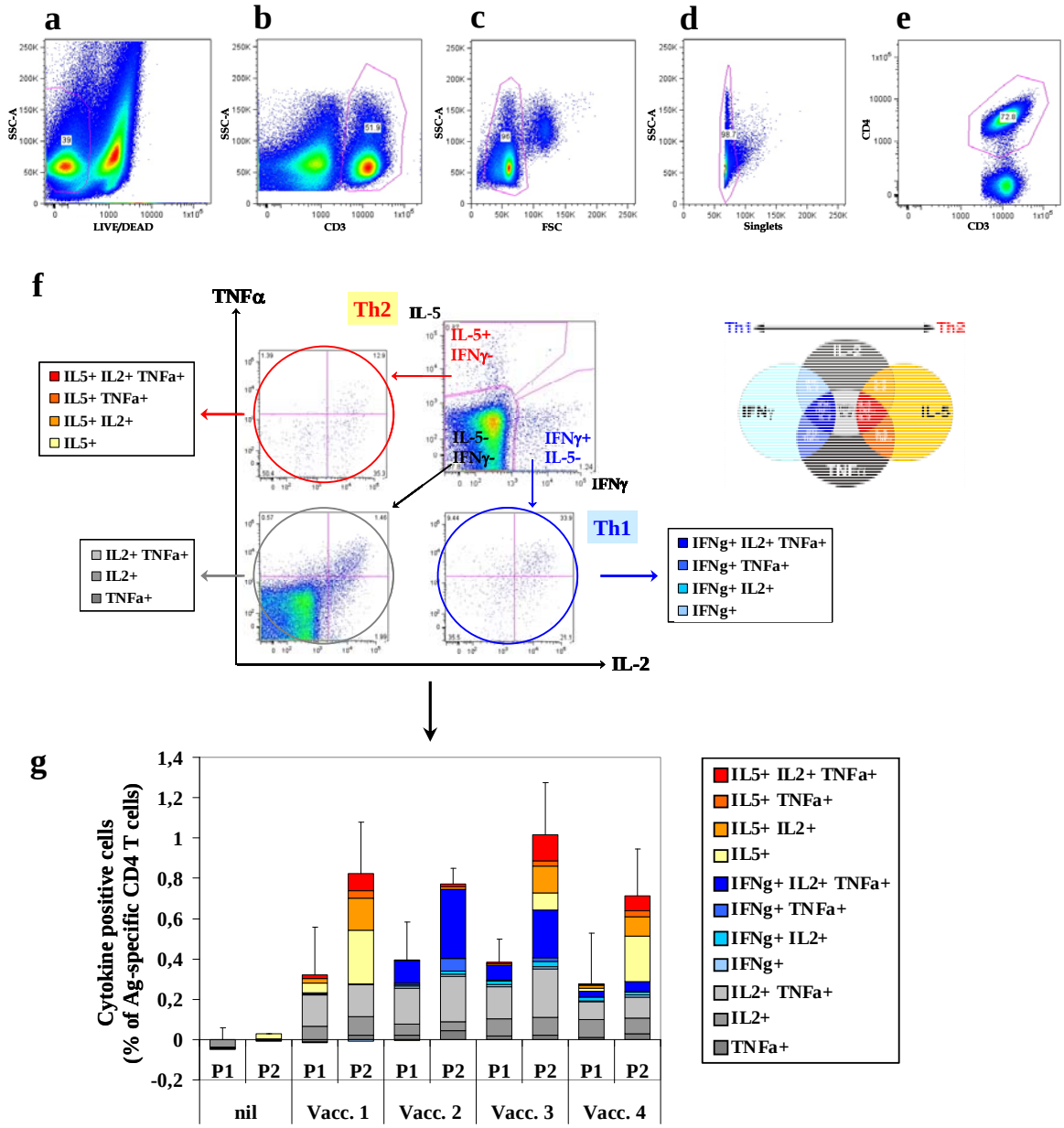


Fig. 1: Evaluation of T cell response to influenza vaccine: FACS analysis of in-vitro restimulated splenocytes.

Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with trivalent influenza vaccine (composed of equal amounts of haemagglutinin from influenza strains H1N1 A, H3N2 A and B) either alone or adjuvanted. At 2 weeks after 1st and 2nd immunization immunization mouse spleens were taken, splenocytes were re-stimulated in vitro with the vaccine proteins and intracellular cytokine staining was performed.

In flow cytometry analysis, dot plots are generated to select cells, by choosing the appropriate gates, on the basis of cell viability (**Fig. 1a**), morphological characteristics (**Fig. 1c**), the aggregates are excluded by gating on the singlet population (**Fig. 1d**). Lymphocytes are discriminated on the basis of expression of surface molecule CD3 (**Fig. 1b**) and specific T helper lymphocyte subpopulation for expression of both CD3 and CD4 surface markers (**Fig. 1e**). Through appropriate gating, CD4+ T cells expressing single cytokines (IFNγ, IL-5, TNFα, IL-2) or combinations thereof are identified (**Fig. 1f**). In **Fig. 1g** histograms show CD4+ T cell responses 2 weeks post-1 and second dose of the vaccine, estimated by the frequency of CD4+ T cells producing cytokines.

Each bar represents the mean of the response of splenocytes from three spleens. A total of three experiments with similar outcome were performed.

F**ig. 1** shows a representative example of how the analysis of T cell responses to Flu vaccines was performed. Balb/c mice were immunized twice, 3 weeks apart, with trivalent subunit Flu vaccine (composed of equal amounts of haemagglutinin from influenza strains H1N1 A, H3N2 A and B) alone or adjuvated. At 2 weeks post-1 and 2nd immunization, mouse spleens and sera were taken and analyzed respectively for CD4+ T cell and antibody responses.

Splenocytes were stimulated in vitro with one or all three antigens used in the vaccine and Ag-specific CD4+ T cells were analyzed for cytokine production by flow cytometry after intracellular cytokine staining (ICS), as described in materials and methods.

Through appropriate gating (**Fig. 1a-f**), CD3+CD4+ cells expressing single cytokines (IFNγ, IL-5, TNFα, IL-2) or combinations thereof can be identified and further distinguished in two subpopulations: the indicator cytokines for Th1 response is IFNγ, for Th2 response is IL-5, and a color coding was used to show the fraction of cells expressing either one or the other of these cytokines. No IL-5/IFNγ double positive cells were found in these experiments. Finally, the overall frequency of CD4+ T cells producing cytokines is represented in multicolor bar graphs (**Fig. 1g**), with each bar showing the mean of the response of splenocytes from three spleens.

The protective effect of HA-specific antibodies has been demonstrated both in experimentally infected animals and humans [174]. For this reason, the induction of HA-specific antibodies is used as a correlate of vaccine efficacy. To assess humoral response to Flu vaccines, HA-specific serum antibody titres were measured by ELISA on mouse sera collected 2 weeks after the 1st and the 2nd immunization (**Fig. 2**).

Total IgG titres against all three vaccine strains were measured 2 weeks post-1 and 2nd vaccination (**Fig. 2a-c**), whereas serum IgG subclasses were calculated 2 weeks after 2nd immunization (**Fig. 2d**). IgG1 and IgG2a isotypes are known to be associated respectively with type-2 and type-1 immune responses.

Given that IgG2a production is indicative of a T helper 1 response and IgG1 production is indicative of a T helper 2 response, the cellular and humoral immune responses to different vaccination regimens were compared qualitatively. The ratios between antibody titres IgG2a:IgG1 and the frequency of IFNγ:IL-5 producing T cells were calculated and represented in the same graph (**Fig. 3**).

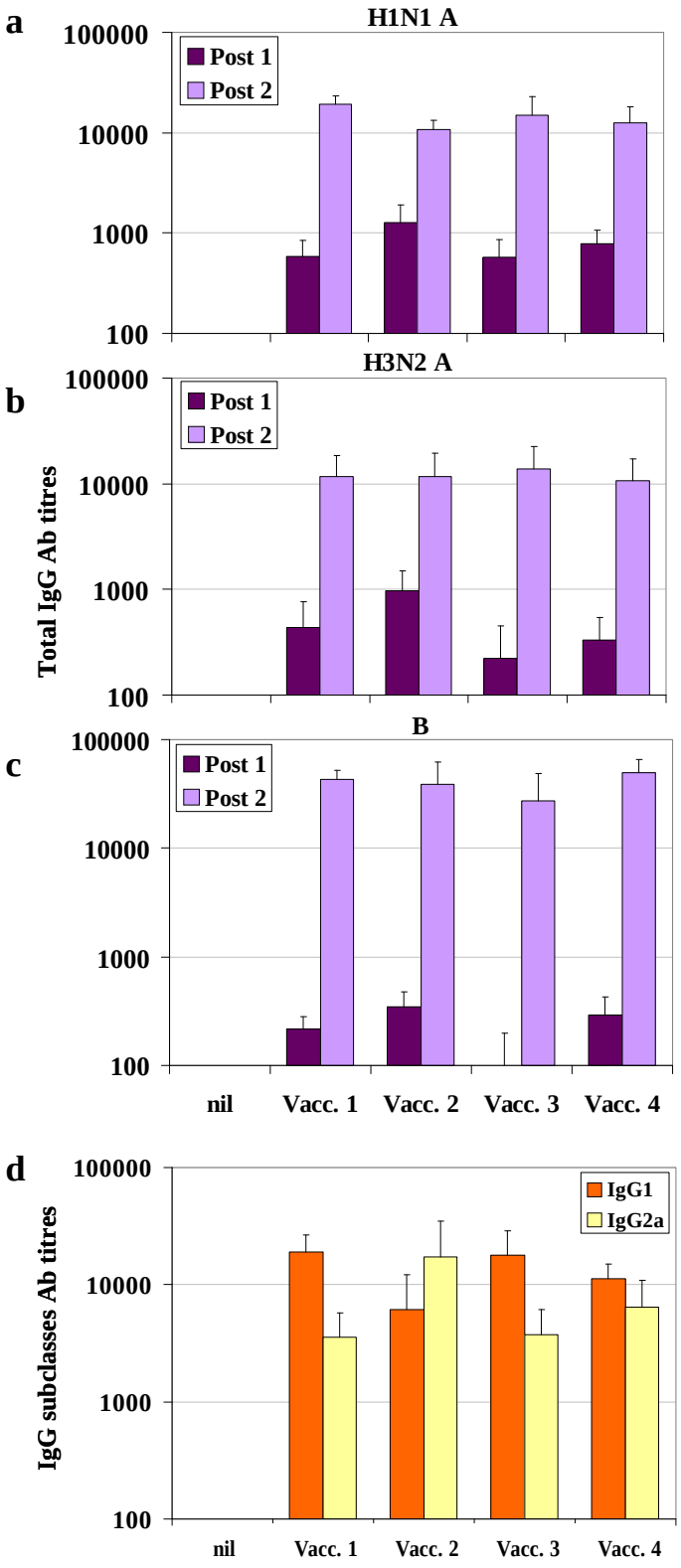


Fig. 2: Evaluation of humoral response to influenza vaccine: total IgG and IgG subclasses serum titres. Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with trivalent influenza vaccine (composed of equal amounts of hemagglutinin from influenza strains H1N1 A, H3N2 A and B) either alone or adjuvanted. At 2 weeeks after 1st and 2nd immunization mouse sera were collected. Shown are the geometric means (and standard error) of serum IgG titres against H1N1 A (**Fig. 2a**), H3N2 A (**Fig. 2b**) and against B (**Fig. 2c**) 2 weeks post-1 (8 mice/group) and second dose (5 mice/group). **Fig. 2d** shows HA-specific IgG1 and IgG2a titres (geometric mean titres and standard error) against H1N1 A measured by ELISA on mouse sera 2 weeks post 2nd immunization.

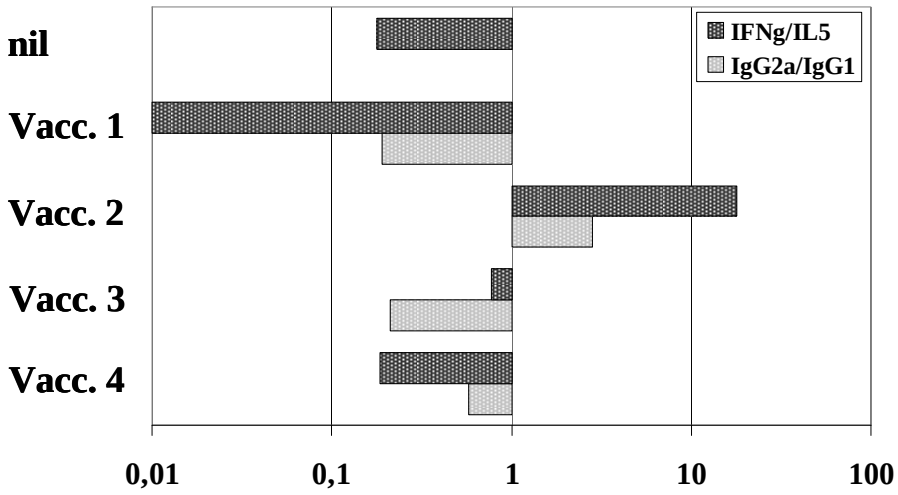


Fig. 3: Correspondence between cellular and humoral immune responses to influenza vaccine: ratios type 1/type 2 immune responses.
Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with trivalent influenza vaccine either alone or adjuvanted. At 2 weeeks after 2nd immunization immunization mouse spleens and sera were taken and analyzed for T cell and antibody responses. Darker bars (▩) represent the ratios of IFNγ positive divided by IL-5 positive CD4+ T cells. Lighter bars (▨) represent the titre ratios of IgG2a:IgG1 isotype geometric mean titres against H1N1 A.

In the present study, cellular and humoral immune responses to subunit influenza vaccines formulated with different adjuvant combinations were investigated, with the aim of evaluating new vaccines. In the following, a detailed biological analysis of immune responses to these novel vaccines is presented.

Adjuvants are frequently added to antigens in vaccine formulations to improve immune responses. MF59 has been shown to be particularly effective for enhancing antibody and T cell responses [159]. However, it is not a potent adjuvant for the induction of Th1 cellular immune responses, which may be required to provide protective immunity against influenza virus. Several experimental Th1 immune-potentiators, including TLR agonists, are in development and have been shown to have profound effects on vaccine potency.
Here we evaluated MF59 for its ability to improve Flu vaccine immunogenicity and efficacy and a range of alternative adjuvants (the TLR9 agonist CpG, the TLR4 agonist E6020 and the TLR9 agonist IC31) alone or co-delivered with MF59 emulsion, as potential new generation approaches.

4.2 MF59 potentiates Th2-biased humoral and cellular immune responses to Flu antigens as well as their shift towards a Th1 type after CpG addition

The TLR9 agonist CpG is known to act as potent vaccine adjuvant in a range of species and for a number of vaccines [152 and 175]. Additionally, it has been recently demonstrated that its combination with MF59 strongly biases the immune response to Flu vaccine toward a type-1 profile [137].

The Ag-specific T cell stimulation assay (**Fig. 4a**) clearly shows that MF59 is the most potent single adjuvant for the induction of T cell response to trivalent Flu vaccine, strongly enhancing its magnitude, especially post 2^{nd} immunization, without altering its quality, i.e. the composition of cytokines produced, found already in the IL-5 dominated response (Th2) induced by vaccine alone. The CpG adjuvant alone is not very effective, inducing only a weak Th1 response, dominated by IFNγ production. The addition of CpG to MF59 does not increase the overall magnitude of the T cell response, but induces its shift towards a Th1-type. In **Fig. 4a** it is also evident that a booster dose of vaccine 3 weeks after the first one increases significantly the frequency of Ag-specific T cells producing cytokines, mainly in mice receiving vaccine + MF59 and vaccine + MF59 + CpG, but the quality of T cell response established post-1 immunization does not change.

Fig. 4b-e shows the assessment of serum HA-specific total IgG (**Fig. 4b-d**), IgG1 and IgG2a (**Fig. 4e**) antibody titres. **Fig. 4b-d** demonstrate that, also for humoral responses, MF59 is the most potent single adjuvant, inducing significantly enhanced total IgG titres post-1 and post-2 doses for each of the three antigens derived from influenza strains included in the vaccine. For H1N1 A, H3N2 A and B strains, MF59 induces an 18-, 3- and 15-fold increase respectively in post-2 doses total IgG titres, while CpG induces a total IgG production post-2 doses comparable (for H1N1 A and B strains) or even lower (for H3N2 strain) than vaccine alone. The addition of CpG to MF59 enhances the total IgG titres post-1^{st} and 2^{nd} dose, as well as vaccine + MF59, compared to vaccine alone and vaccine + CpG. Even if the magnitude of humoral responses is similar between MF59 and MF59 + CpG adjuvanted Flu vaccines, its quality is completely different. IgG isotyping was performed to determine the levels of IgG1 (indicative of a Th2-type response and expected from MF59) and IgG2a (indicative of a Th1-type response and expected from CpG) post 2^{nd} immunization (**Fig. 4e**).

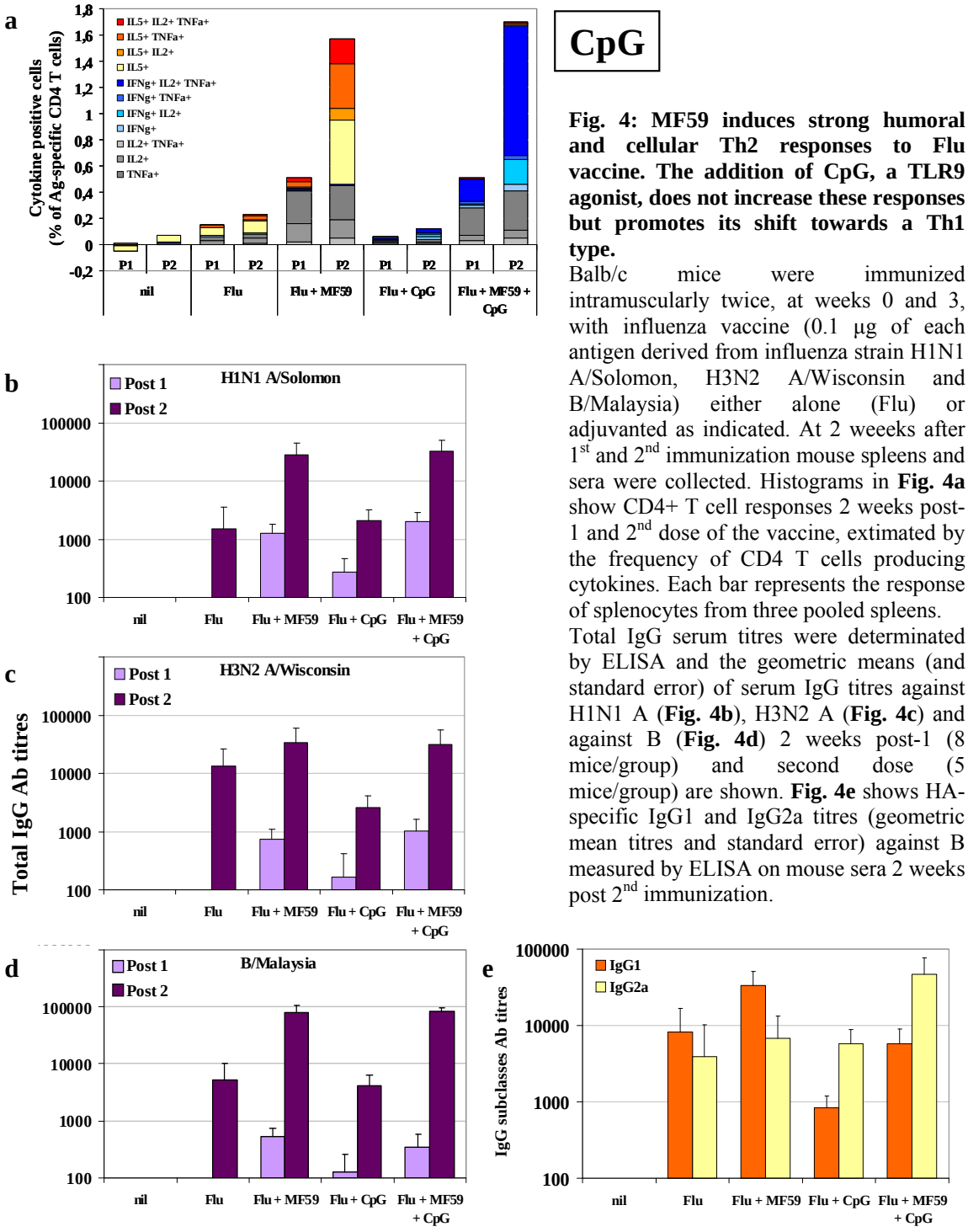


Fig. 4: MF59 induces strong humoral and cellular Th2 responses to Flu vaccine. The addition of CpG, a TLR9 agonist, does not increase these responses but promotes its shift towards a Th1 type.

Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 µg of each antigen derived from influenza strain H1N1 A/Solomon, H3N2 A/Wisconsin and B/Malaysia) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 1st and 2nd immunization mouse spleens and sera were collected. Histograms in **Fig. 4a** show CD4+ T cell responses 2 weeks post-1 and 2nd dose of the vaccine, extimated by the frequency of CD4 T cells producing cytokines. Each bar represents the response of splenocytes from three pooled spleens.
Total IgG serum titres were determinated by ELISA and the geometric means (and standard error) of serum IgG titres against H1N1 A (**Fig. 4b**), H3N2 A (**Fig. 4c**) and against B (**Fig. 4d**) 2 weeks post-1 (8 mice/group) and second dose (5 mice/group) are shown. **Fig. 4e** shows HA-specific IgG1 and IgG2a titres (geometric mean titres and standard error) against B measured by ELISA on mouse sera 2 weeks post 2nd immunization.

The addition of CpG to vaccine alone or to vaccine + MF59 promotes the shift of humoral response from an IgG1- to an IgG2a-dominated isotype, clearly type-1 biased. Instead, MF59 increases antibody response to vaccine without modifying its quality, type-2 biased, as reflected by IgG1 dominance (**Fig. 4e**).

The qualitative correspondence between cellular and humoral immune responses is more evident in **Fig. 5**, in which the ratios between the frequency of IFNγ:IL-5 producing T cells and IgG2a:IgG2a antibody titres are compared.

The primary antiviral activity of CD4+ T cells in adaptive immunity is to provide help to B cells to produce antiviral antibodies [141]. Several CD4+ T cell-derived cytokines modulate isotype switching of antibodies. The production of IgG1 in mice is dependent on the presence of Th2 cytokines (i.e. IL-4 and IL-5), whereas the switch to IgG2a antibodies is mediated via Th1 cytokines (i.e. IFNγ). This dichotomy is well reflected here (**Fig. 5**), where higher IgG2a:IgG1 antibody ratios are associated with increased IFNγ production compared to IL-5 (in the case of the addition of CpG to vaccine alone or adjuvanted with MF59). Vice versa, vaccine and vaccine + MF59 promote a type-2 immune response both at humoral and cellular levels, inducing low IgG2a antibody titres (lower IgG2a:IgG1 ratios) associated with low IFNγ production.

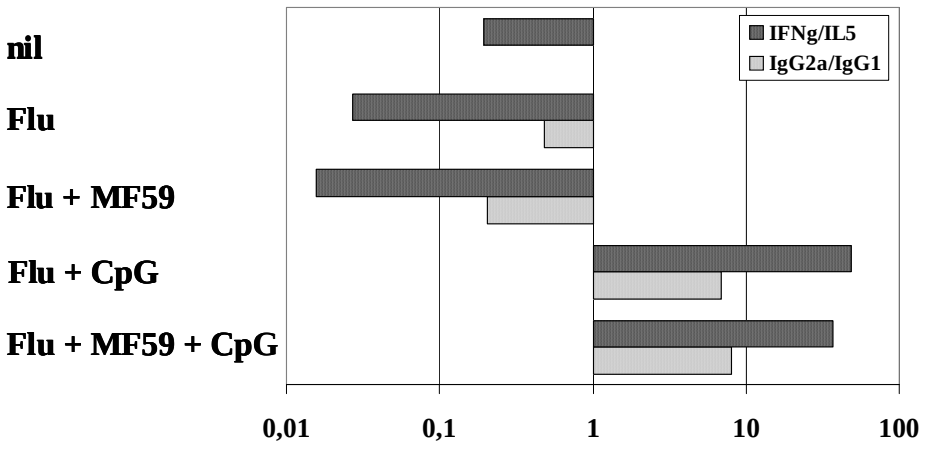


Fig. 5: MF59 potentiates both type-1 and type-2 biased immune responses maintaining the type 1/type 2 ratios almost unchanged.
Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 μg of each antigen derived from influenza strain H1N1 A/Solomon, H3N2 A/Wisconsin and B/Malaysia) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 2nd immunization immunization mouse spleens and sera were taken and analyzed for T cell and antibody responses. Darker bars (▩) represent the ratios of IFNγ positive divided by IL-5 positive CD4+ T cells. Lighter bars (□) represent the titre ratios of IgG2a:IgG1 isotype geometric mean titres against B.

4.3 Ability of immune-potentiators E6020 and IC31 to modulate the quality of immune responses to Flu vaccine alone or adjuvanted with MF59

In the current study, other two immune-potentiators (TLR agonists), given alone or co-delivered with MF59 emulsion, were evaluated for their ability to enhance protective immunity to subunit influenza vaccine, and their ability to modulate the quality of the immune response by shifting it towards a more Th1 biased response. The selected immune-potentiators include E6020, a TLR4 agonist, and IC31, a TLR9 agonist.

E6020, a structural mimic of lipid A (the simplest active forms of lipopolysaccharide), is a synthetic substance that was discovered at the Eisai Research Institute of Boston. This TLR4 agonist, eliciting an immunostimulatory response, could be used as vaccine adjuvant [176].

IC31 (Intercell, Vienna, Austria) is a novel two-component adjuvant which combines two immunomodulatory compounds: an antibacterial peptide (11-mer cationic peptide KLKL5KLK) and a TLR9 agonist, a synthetic ODN1a, without a CpG motif [177]. Experiments performed in adult mice showed increased humoral and cellular immune responses to seasonal influenza vaccines [141].

The Ag-specific T cell response in **Fig. 6a** shows that the addition of E6020 alone to vaccine antigens promotes a Th1 response, inducing IFNγ production by CD4+ T cells, but does not increase the overall magnitude of T cell response generated by non-adjuvanted vaccine. The addition of E6020 to MF59 induces a higher response post-1 and a shift toward a Th1 profile. In the post-2 response, MF59 + E6020 induce both IFNγ and IL-5 producing T cells, which represents a cytokine profile that is more balanced between Th1 and Th2.

Fig. 6b shows the adjuvantation with IC31 at two doses (high and low) alone or in combination with MF59. IC31 alone, both high (IC31h) and low (IC31l), significantly increases the level of Ag-specific T cells, especially IFNγ producing T cells and in particular post 2^{nd} immunization. Therefore it drives a shift of cellular response to Flu antigens from a Th2 to a Th1 profile, even if CD4+ T cells producing IL-5 are still detected. This mixed Th1/Th2 response is more evident when IC31 is added to vaccine formulations containing MF59. Again the combination of MF59 with a TLR agonist enhances significantly Ag-specific CD4+ T cell cytokine production compared to vaccine

alone and vaccine + IC31 (high and low), at similar levels as MF59-adjuvanted vaccine; however, the profile of produced cytokines is much more balanced between Th1

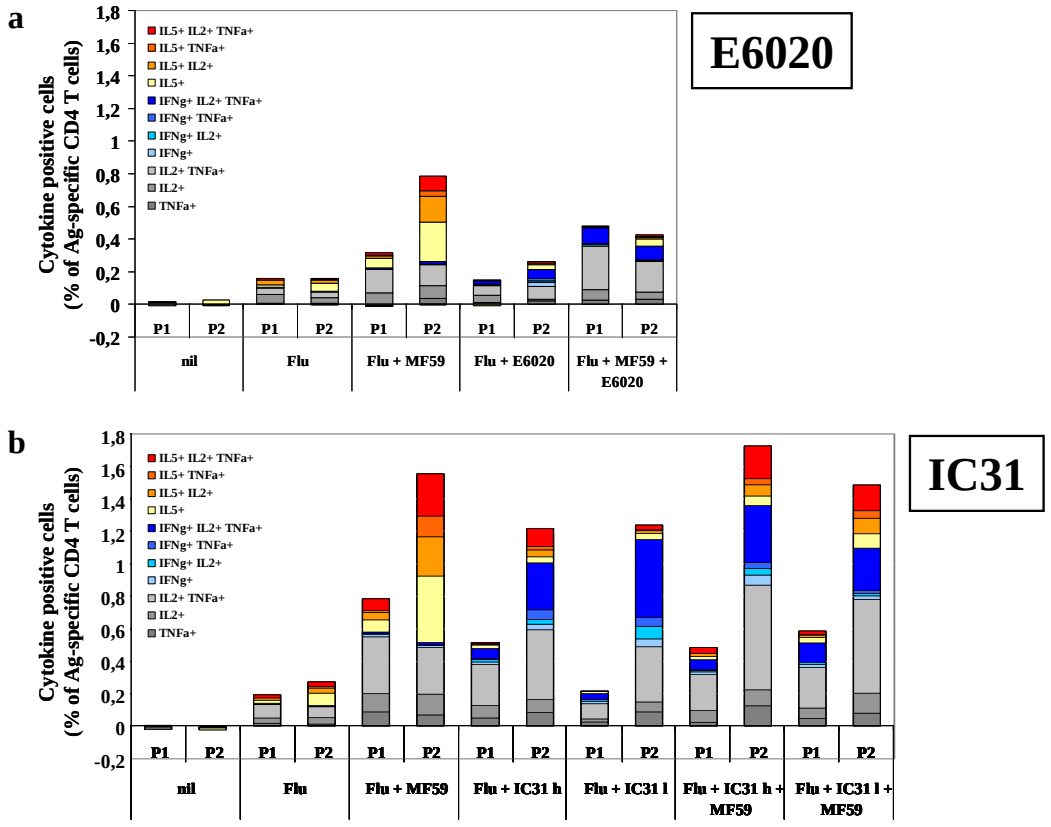


Fig. 6: The combination of MF59 with E6020, a TLR4 agonist, shifts CD4+ T cell response towards a Th1-type, whereas the addition of IC31, a TLR9 agonist, promotes a mixed Th1/Th2 cellular response.
Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 µg of each antigen derived from influenza strain H1N1 A/Solomon, H3N2 A/Wisconsin and B/Malaysia) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 1st and 2nd immunization mouse spleens were collected. Histograms in **Fig. 6a** and **b** show CD4+ T cell responses 2 weeks post-1 and 2nd dose of the vaccine, extimated by the frequency of CD4+ T cells producing cytokines. Each bar represents the response of splenocytes from three pooled spleens. A total of three experiments with similar outcome were performed.

and Th2 compared to single adjuvants (MF59, IC31h, IC31l) that promote a more polarized Th response.

We also investigated the influence of E6020 and IC31 on the induction of HA-specific antibody levels (**Fig. 7**).

After one immunization, E6020 is less potent compared to MF59 adjuvanted vaccine with respect to antibodies induced against all three vaccine strains (**Fig. 7a-c**). However, after 2 vaccine doses significant differences between E6020 and MF59 adjuvanted vaccines are

not detectable for total IgG antibody titres. The co-delivery of E6020 with MF59 adjuvanted Flu vaccine does not lead to a significant increase of total IgG antibody titres, but is comparable to titres induced by MF59 alone. Interestingly, total IgG titres

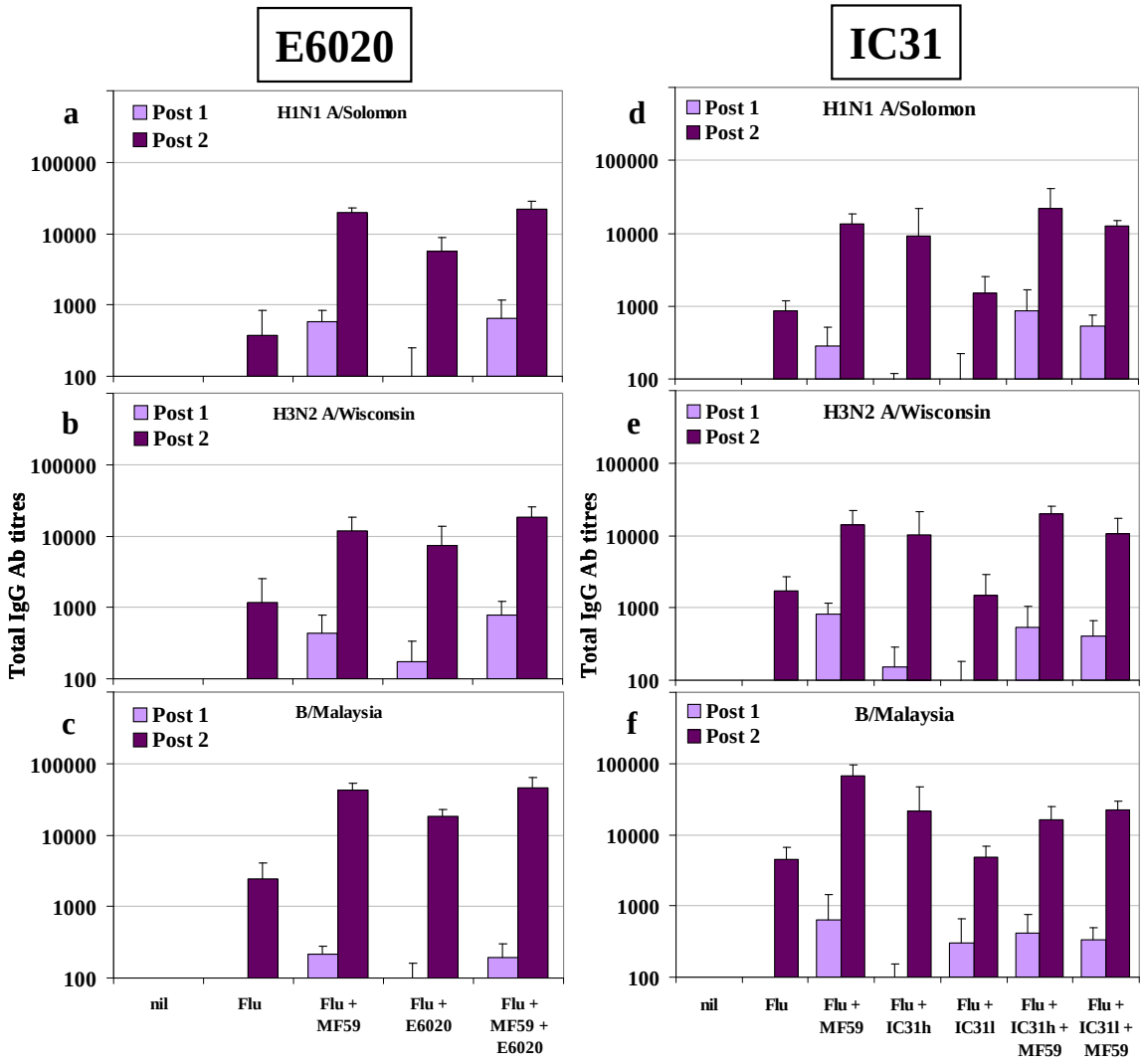


Fig. 7: Addition of E6020 or IC31 to MF59 not greatly increases total IgG antibody response to MF59 adjuvanted Flu vaccine.
Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 µg of each antigen derived from influenza strain H1N1 A/Solomon, H3N2 A/Wisconsin and B/Malaysia) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 1st and 2nd immunization mouse sera were collected. Total IgG serum titres were determinated by ELISA and the geometric means (and standard error) of serum IgG titres against H1N1 A (**Fig. 7a** and **d**), H3N2 A (**Fig. 7b** and **e**) and against B (**Fig. 7c** and **f**) 2 weeks post-1 (8 mice/group) and second dose (5 mice/group) are shown.

post-2 anti H1N1 A/Solomon induced by non-adjuvanted Flu vaccine are lower when compared to all given adjuvanted formulations (**Fig. 7a**), so that the adjuvant effect of

MF59, E6020 and their combination is more evident for this strain rather than for the other two.

As for E6020, also both high and low doses of IC31 induce lower IgG titres than MF59 adjuvanted vaccine post-1 immunization (**Fig. 7d-f**). After 2 vaccine doses, comparable levels of total IgG are detected between MF59 and IC31h adjuvanted vaccines for H1N1 A/Solomon and H3N2 A/Wisconsin strains, whereas IC31h induces lower antibody titres anti B/Malaysia than MF59. Instead, IC31l is significantly less potent in inducing IgG titres against all three vaccine strains compared to MF59 and IC31h adjuvanted vaccine. The combination of IC31h and IC31l with MF59 does not increase greatly the total IgG response with respect to MF59 alone for H1N1 A/Solomon and H3N2 A/Wisconsin, and even decreases it for B/Malaysia strain compared to MF59 or IC31h alone. The magnitude of total IgG response against all three vaccine strains post 2nd immunization is dose-dependent with respect of adjuvantation with IC31. Interestingly, if the TLR agonist is added to MF59 this effect is much less evident for H1N1 A/Solomon and H3N2 A/Wisconsin (**Fig. 7e-f**) and disappears for B/Malaysia (**Fig. 7g**).

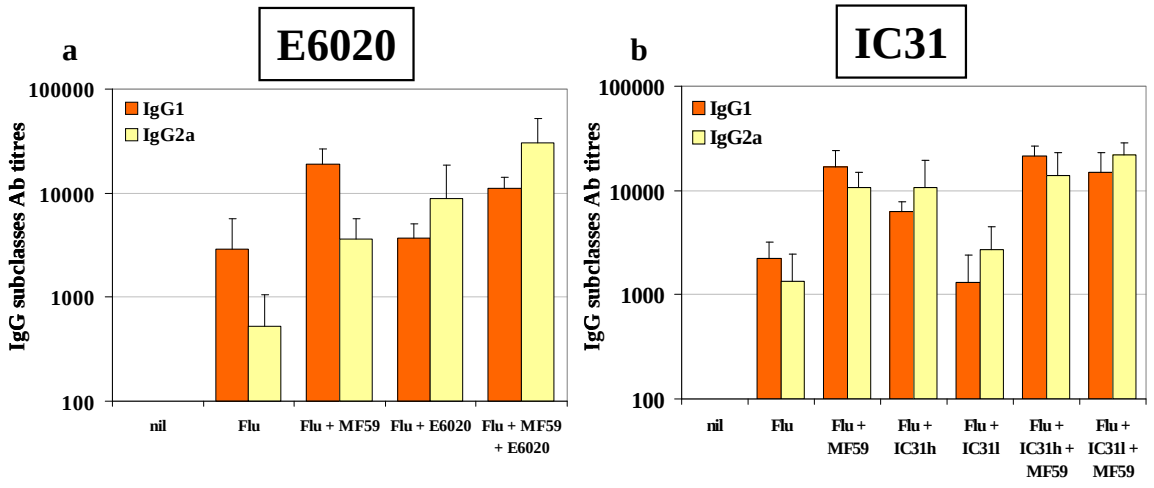


Fig. 8: Co-delivery of E6020 or IC31 with MF59 increases IgG2a antibody titres.
Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 μg of each antigen derived from influenza strain H1N1 A/Solomon, H3N2 A/Wisconsin and B/Malaysia) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 1st and 2nd immunization mouse sera were collected. **Fig. 8 a** and **b** show HA-specific IgG1 and IgG2a titres (geometric mean titres and standard error) against B measured by ELISA on mouse sera 2 weeks post 2nd immunization.

As expected, the addition of E6020 to vaccine alone or to MF59 adjuvanted vaccine enhances B/Malaysia-specific IgG2a antibody isotype post 2nd immunization (**Fig. 8a**), in agreement with previous observed Th1 activation (**Fig. 6a**). Interestingly the addition of

MF59 to vaccine either formulated without immune-potentiator or with E6020 does not influence the quality of the immune response, but significantly enhances both IgG1 and IgG2a isotype antibody titres, maintaining its type-2 profile for vaccine and its type-1 profile for E6020 adjuvanted vaccine (**Fig. 8a**).

Regarding the TLR9 agonist IC31, at higher dose it increases both B/Malaysia-specific IgG1 and IgG2a antibody titres, compared to vaccine alone, eliciting much more IgG2a isotype. In contrast, at lower dose it only promotes the shift of the humoral response to vaccine from type-2 to type-1 (dominated by IgG2a isotype), without modifying its overall magnitude (**Fig. 8b**). Surprisingly, the addition of MF59 to IC31 adjuvanted vaccine, although it elicits both IgG subclass levels, does not change the quality of immune response at lower dose of IC31, whereas, when added to IC31h, promotes its shift toward a more type-2 profile. Thus, IC31 induces a more balanced type-1/type-2 of humoral immune response than the other immune-potentiators evaluated here.

According to the data shown in **Fig. 6a** and **Fig. 8a**, the ratios between type-1:type-2 of cellular and humoral immune responses post 2^{nd} immunization indicate that there is a qualitative correspondence between cytokines and IgG subclasses produced for E6020 adjuvanted vaccine (**Fig. 9a**). In the presence of E6020 there is an increase of IFNγ:IL-5 ratio as well as IgG2a:IgG1 ratio, although this shift of immune response toward a type-1 profile is less evident than in the case of CpG adjuvant (**Fig. 5**), indicating that E6020 generate a more balanced type-1/type-2 response, rather than skewing to either extreme.

The situation is more complex for the IC31 adjuvant (**Fig. 9b**): its addition to vaccine alone shifts the immune response toward a more type-1 profile; when it is co-delivered with MF59, the profile of immune response become much more type-1/type-2 mixed for cellular and humoral responses, losing the correspondence between cytokine and antibody isotype production for both IC31 doses. These data also indicate that the shift of immune response toward a more pronounced type-1 profile is not dose-dependent, because in the MF59 adjuvanted vaccine formulated with IC31l, IgG2a:IgG1 ratio is higher than in the MF59 adjuvanted vaccine formulated with IC31h (and the opposite happens for T cell response).

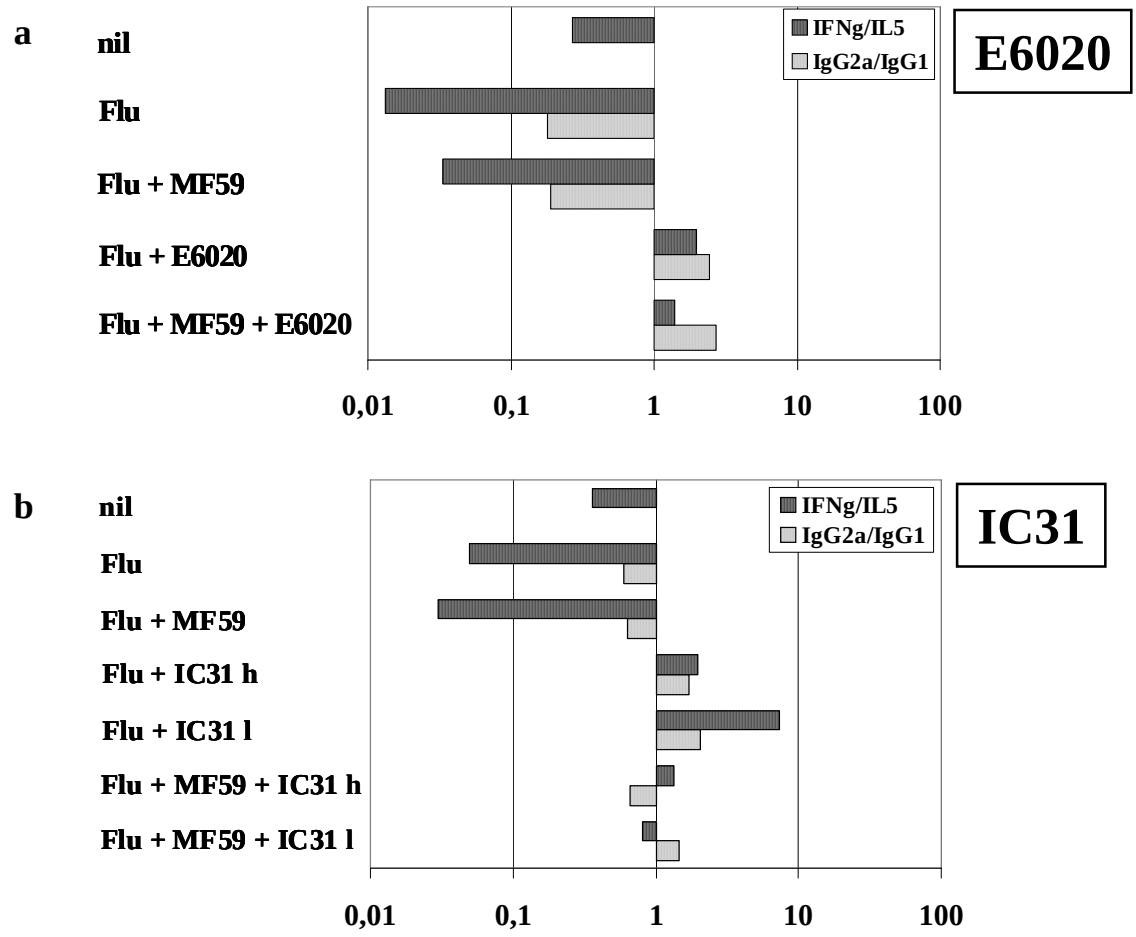


Fig. 9: More balanced type 1/type 2 immune responses induced by TLR agonists E6020 and IC31.
Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 μg of each antigen derived from influenza strain H1N1 A/Solomon, H3N2 A/Wisconsin and B/Malaysia) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 2nd immunization immunization mouse spleens and sera were taken and analyzed for T cell and antibody responses. Darker bars (▩) represent the ratios of IFNγ positive divided by IL-5 positive CD4+ T cells. Lighter bars (□) represent the titre ratios of IgG2a:IgG1 isotype geometric mean titres against B.

To summarize, we have evaluated here different adjuvants (MF59, CpG, E6020 and IC31), alone and in combinations, for their ability to enhance and modulate antibody and T-cell responses induced by subunit influenza vaccines. The observed results suggest the possibility to drive the immune response into a desired direction by choosing appropriate adjuvant combinations.

Significant amounts of preclinical data on MF59 adjuvant are available and they indicate that this adjuvant typically induces a Th2-type immune response to vaccine in Balb/c mice. So we've focused our attention on CpG immune-potentiator, which it has been demonstrated to induce strong type-1 immunity in combination with MF59.

4.4 MF59 and CpG mediate sustained influenza vaccine-specific cellular immune response

It would be highly advantageous if improved influenza vaccines induced persistent immune responses and memory that could be efficiently boosted in the following influenza season.

To examine the efficacy of subunit influenza vaccine formulated alone or adjuvanted with MF59, CpG and their combination to induce long term response, Balb/c mice were immunized twice 3 weeks apart and cellular immune response was assessed 2 weeks post 1 and 2 weeks and 4 months after the last immunization by measuring the frequency of Ag-specific CD4+ T cells producing cytokines in mouse splenocytes restimulated in vitro (**Fig. 10**).

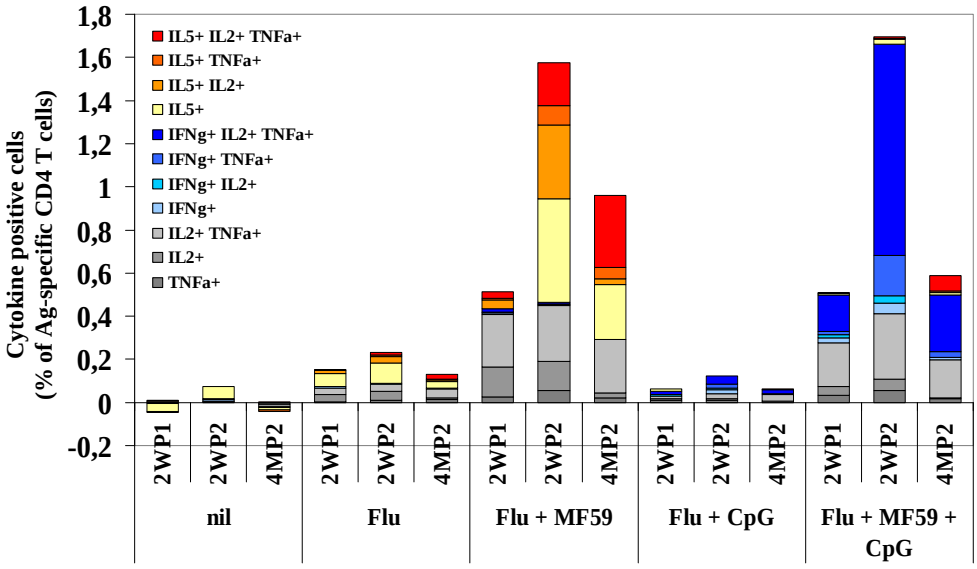


Fig. 10: Long-term persistence of Ag-specific CD4+ T cell response to Flu vaccine.
Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 µg of each antigen derived from influenza strain H1N1 A/Solomon, H3N2 A/Wisconsin and B/Malaysia) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 1st and 2nd immunization and at 4 months after 2nd immunization mouse spleens were collected. Histograms show CD4+ T cell responses 2 weeks post 1 (2WP1) and 2 weeks (2WP2) and 4 months (4MP2) post 2 doses of the vaccine, extimated by the frequency of CD4+ T cells producing cytokines. Each bar represents the response of splenocytes from three pooled spleens.

A sustained cellular immune response is well detected also 4 months post 2nd immunization and the quality of this response remains stable during this time. Even at this late time-point, adjuvants mediate an increase in vaccine-specific T cell responses, although to a lower extent than observed 2 weeks post 2nd immunization. Interestingly, the cellular response induced by MF59 + CpG adjuvanted vaccine decreases during the time more quickly than that induced by MF59 adjuvanted vaccine. In all cases, CpG strongly shifts the immune response towards a type-1 profile, whereas vaccine alone and with MF59 promotes long-lasting type-2 immunity, in accordance with the data from 2 weeks post-2. Thus, it seems clear that MF59 adjuvant and its combination with CpG improve subunit influenza vaccine to induce potent long-term immune responses. These sustained responses are expected to be more protective against influenza infection that occurs months after immunizations than those induced by non-adjuvanted vaccine.

4.5 Th1 shift induced by CpG is dose dependent

To evaluate if adjuvant effects of CpG on immune response to Flu vaccine are dose-dependent and to find the optimal CpG dose, a range of different concentrations (25, 10, 1 and 0.1 μg/mouse) of this immune-potentiator was tested in Balb/c mice in combination with vaccine alone or adjuvanted with MF59. Cellular and humoral immune responses were analysed 2 weeks after 2nd immunization (**Fig. 11**).

Fig. 11a shows Ag-specific CD4+ T cell responses after in vitro restimulation with vaccine antigens. Treatment with CpG alone promotes a clear shift of immune response to vaccine from a Th2 to a Th1 type, dominated by IFNγ production, at all concentrations used. Nevertheless, this response is rather weak with respect to vaccine alone and disappears at 0.1 μg/mouse. The addition of CpG to MF59 adjuvanted vaccine induces similar levels of CD4+ T cells producing cytokines compared to vaccine + MF59, with a substantial reduction only at the lowest CpG concentration. Interestingly, the IFNγ dominated response after immunization with CpG decreases by lowering the dose of CpG in presence of MF59, switching back to a Th2 type in a dose-dependent manner.

Analysis of serum total IgG antibody titres (**Fig. 11b**) shows that the three vaccine strains differently stimulate antibody production: anti B/Jiangsu antibody titres are higher than those induced by the other two strains and H1N1 A/New Caledonia generally induces the

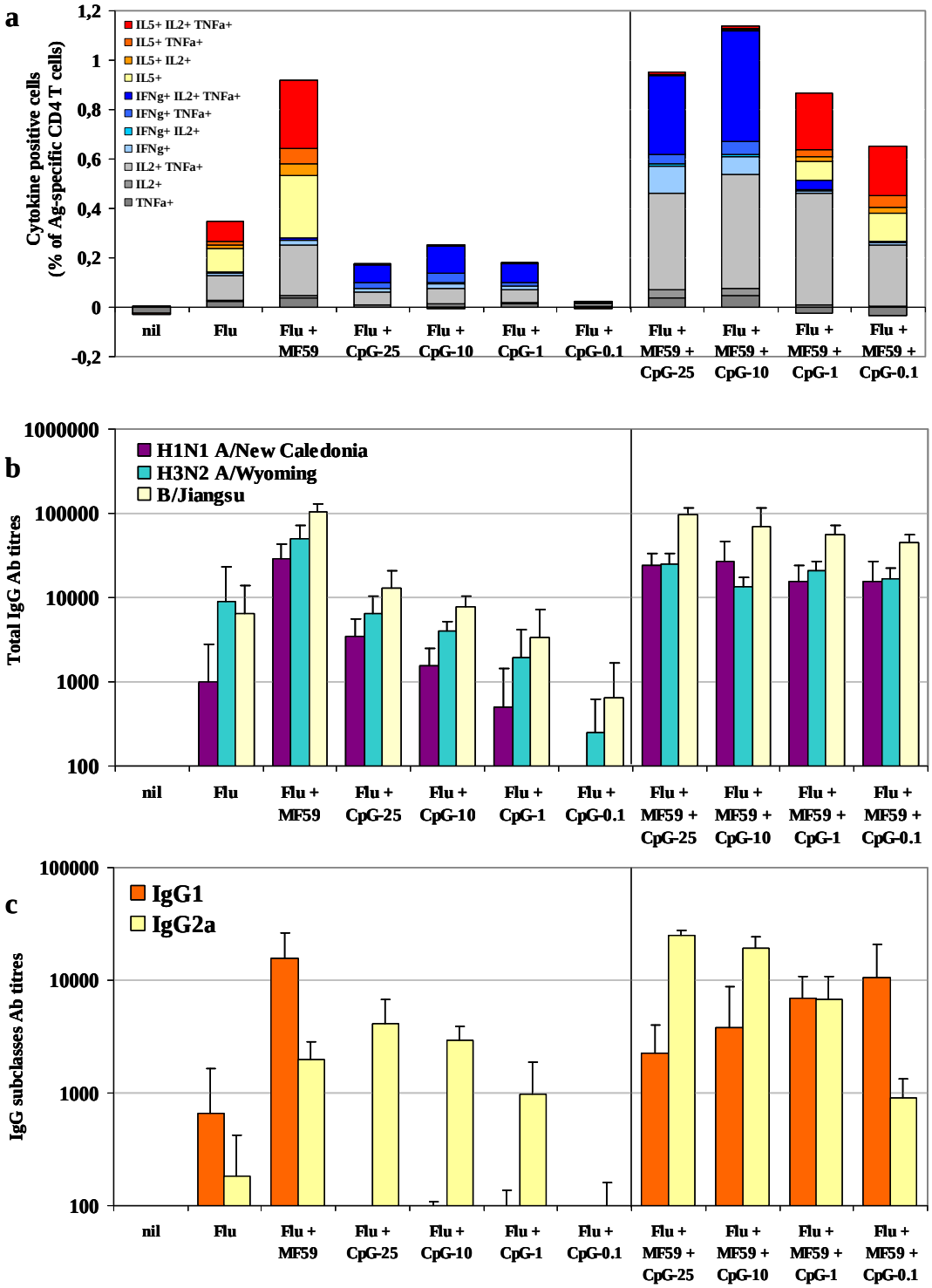


Fig. 11: Th1 shift of cellular and humoral immune response to Flu vaccine induced by CpG addition to MF59 is dose-dependent.

Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 µg of each antigen derived from influenza strain H1N1 A/New Caledonia, H3N2 A/Wyoming and B/Jiangsu) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 2nd immunization mouse spleens and sera were collected. Histograms in **Fig. 11a** show CD4+ T cell responses 2 weeks post 2nd dose of the vaccine, extimated by the frequency of CD4+ T cells producing cytokines. Each bar represents the response of splenocytes from three pooled spleens. Total IgG serum titres were determinated by ELISA and the geometric means (and standard error) of serum IgG titres against H1N1 A, H3N2 A and against B (**Fig. 11b**) 2 weeks post second dose (5 mice/group) are shown. **Fig. 11c** shows HA-specific IgG1 and IgG2a titres (geometric mean titres and standard error) against B measured by ELISA on mouse sera 2 weeks post 2nd immunization.

lowest antibody production. Total IgG titres are decreased when CpG dosage is lowered. MF59 is the most potent single adjuvant, inducing high levels of antibody titres which remain stable in all the formulations. In fact, no significant difference is detectable when a range of CpG concentrations is added to MF59 adjuvanted vaccine.

A qualitative analysis of the humoral response was also performed by measuring IgG isotypes (**Fig 11c**). IgG1 represents the dominant IgG subclass in MF59 adjuvanted vaccine, whereas IgG2a clearly predominates CpG induced humoral responses. The combination of MF59 with CpG promotes higher IgG2a titres than IgG1 at high CpG doses (25 and 10 μg/mouse), but this effect is dose-dependent because by reducing the amount of CpG administered the ratio IgG2a:IgG1 is reverted (**Fig. 12**). This observation is due both to reduction of IgG2a levels and to enhancement of IgG1 levels. These changes in antibody subclasses are paralleled with type-1 and type-2 cytokine profiles of vaccine-specific CD4+ T cells, as shown in **Fig. 12** by the correspondences between IFNγ:IL-5 ratios and IgG2a:IgG1 ratios.

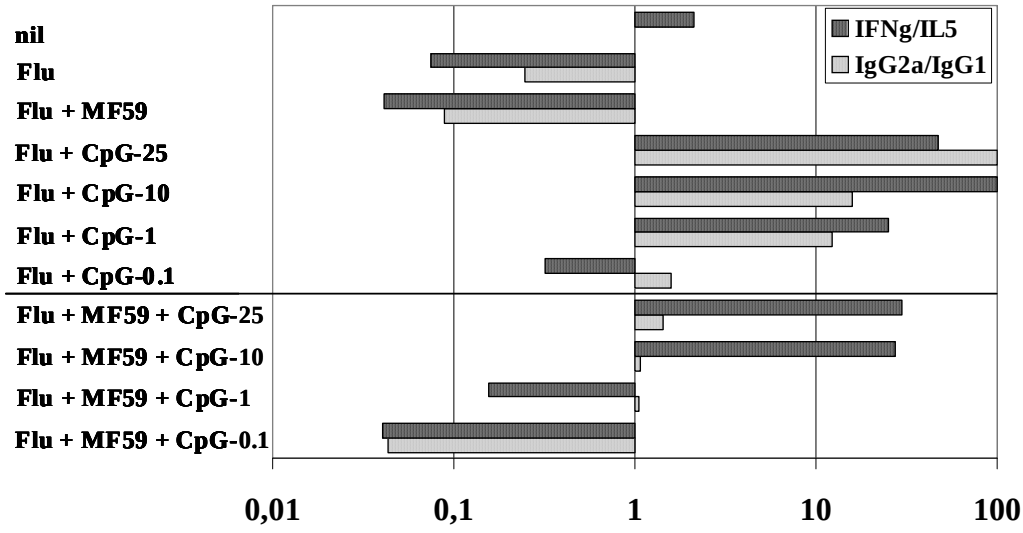


Fig. 12: Both IgG2a:IgG1 and IFNγ:IL-5 ratios decrease in a dose-dependent manner reducing CpG concentration.

Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 μg of each antigen derived from influenza strain H1N1 A/New Caledonia, H3N2 A/Wyoming and B/Jiangsu) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 2nd immunization immunization mouse spleens and sera were taken and analyzed for T cell and antibody responses. Darker bars (▩) represent the ratios of IFNγ positive divided by IL-5 positive CD4+ T cells. Lighter bars (▨) represent the titre ratios of IgG2a:IgG1 isotype geometric mean titres against B.

In conclusion, the dose-dependence of CpG induced effects on influenza vaccines was demonstrated by different immunological parameters: cytokine production by CD4+ T cells (measured by ICS and FACS analysis), and total IgG, IgG1 and IgG2a serum antibody titres (analyzed by ELISA).
Modification of cellular and humoral immune responses by CpG occurs in a dose-dependent manner, achieving a maximum adjuvant effect with 10 μg/mouse.

4.6 Genetic background of mouse strains used in the experiments does not alter the quality of immune response to different vaccine formulations

All data shown up to now have been generated in Balb/c mice, which have an inherent bias toward "Th2 responses" [178]. Subunit influenza vaccine alone and MF59 adjuvanted vaccine have been demonstrated to induce Th2-biased immunity, whereas the addition of CpG to vaccine or to MF59 adjuvanted vaccine promotes a clear switch toward Th1-type immunity. However, it is unclear whether the observed effects are influenced by the genetic predisposition of the mouse strains.
To define whether profiles of immune responses depend on the genetically programmed bias of the mouse strain employed in experiments, the same vaccine formulations were tested in two mouse strains of different genetic backgrounds. Balb/c mice (Th2-prone) and C57BL/6 mice, an alternative mouse strain with less Th2 bias, have been subjected to the same treatments: two immunizations with Flu vaccine alone or adjuvanted with MF59, CpG or their combination. C57BL/6 mice were injected with two different concentrations of vaccine antigens (0.1 and 0.3 μg/ml of each antigen), to establish which was the most effective (because of the lack of data in this regard). At 2 weeks after 2nd immunization spleens and sera were collected to analyze cellular and humoral responses.
The pattern of cytokine production in the two different mouse strains in response to the same treatments is similar (**Fig. 13a**). Ag-specific CD4+ T cells from both Balb/c and C57BL/6 mice vaccinated with vaccine alone and MF59 adjuvanted vaccine produce predominantly Th2-type cytokines (i.e. IL-5). CD4+ T cell response in mice immunized with CpG and CpG + MF59 is dominated by IFNγ production, defining a Th1-type response, both in Th2- and Th1-prone mouse strains.

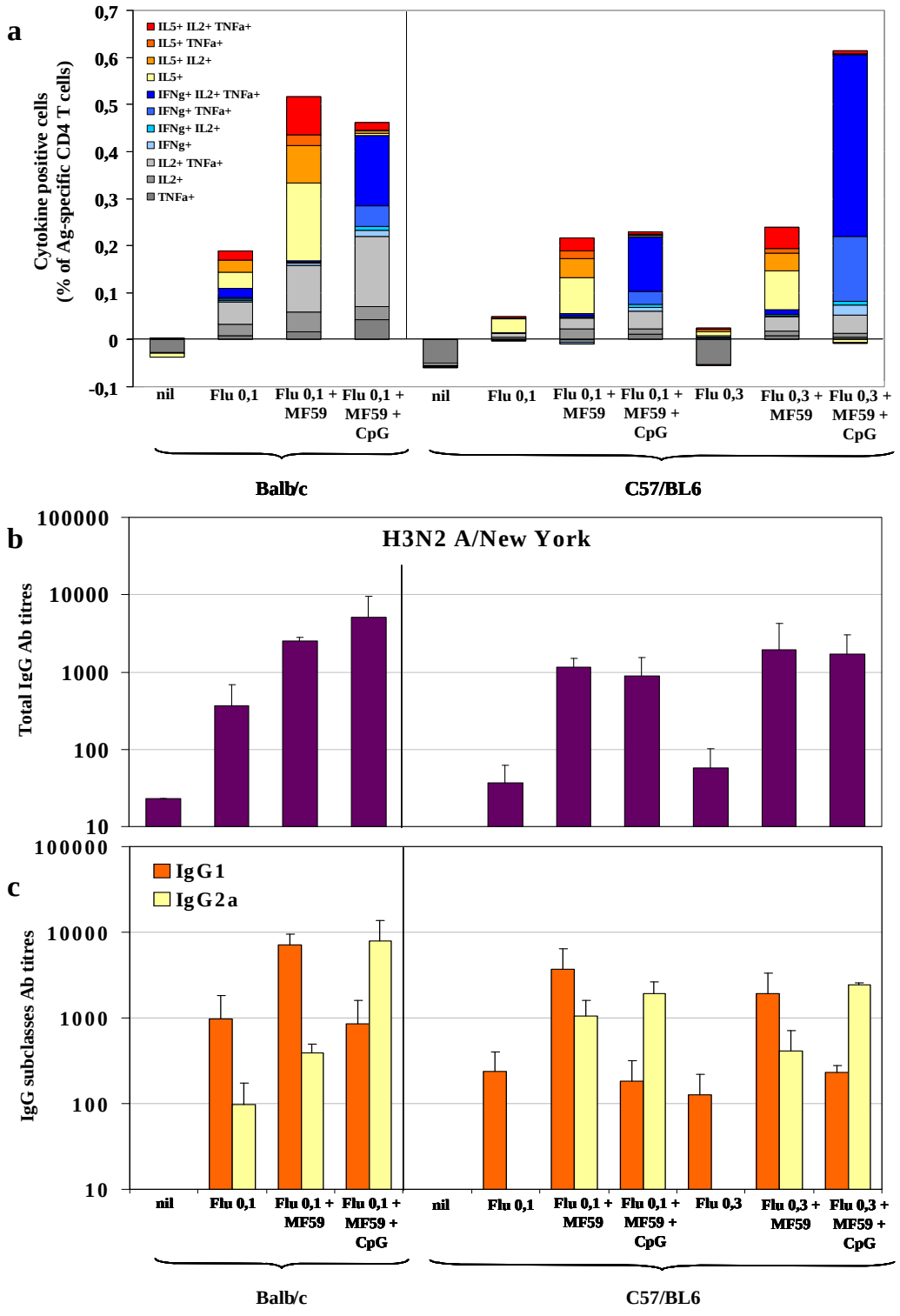


Fig. 13: Similar patterns of cellular and humoral responses to adjuvanted Flu vaccine in different mouse strains (Balb/c and C57/BL6).

Balb/c and C57/BL6 mouse strains were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 µg of each antigen derived from influenza strain H1N1 A/New Caledonia, H3N2 A/New York and B/Jiangsu) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 2nd immunization mouse spleens and sera were collected. Histograms in **Fig. 13a** show CD4+ T cell responses 2 weeks post 2nd dose of the vaccine, extimated by the frequency of CD4+ T cells producing cytokines. Each bar represents the response of splenocytes from three pooled spleens. Total IgG serum titres were determinated by ELISA and the geometric means (and standard error) of serum IgG titres against H3N2 A (**Fig. 13b**) 2 weeks post second dose (5 mice/group) are shown. **Fig. 13c** shows HA-specific IgG1 and IgG2a titres (geometric mean titres and standard error) against H3N2 A measured by ELISA on mouse sera 2 weeks post 2nd immunization.

The levels of the produced cytokines in C57BL/6 mice are generally lower than in Balb/c mice. Only the co-delivery of MF59 and CpG with 0.3 μg/ml of vaccine antigens induces a frequency of IFNγ producing CD4+ T cells higher in C57BL/6 mice with respect to the other strain.

The quality of cellular responses is the same between the two mouse strains and the main difference is related to its magnitude, especially in mice immunized with MF59 adjuvanted vaccine, in which cytokine production is significantly lower in C57BL/6 mice.

Moreover, the overall magnitude of anti-H3N2 A total IgG antibody titres is also reduced in C57BL/6 mice (**Fig. 13b**). Serum IgG antibody levels are similar in C57BL/6 mice vaccinated with MF59 and MF59 + CpG, at 0.1 and 0.3 μg/ml of vaccine antigens, whereas a significant difference was found in the cellular response of the same groups: Ag-specific CD4+ T cell cytokine production is significantly increased in C57BL/6 mice treated with MF59 + CpG at 0.3 μg/ml of Flu antigens compared to C57BL/6 mice immunized with vaccines otherwise adjuvanted (**Fig. 13a**). Interestingly, the adjuvant effect of MF59 alone or in combination with CpG is slightly less marked in Balb/c mice compared to C57BL/6 mice. In fact, in C57BL/6 mice adjuvanted vaccines induces total IgG titres approximately 30 times higher compared to vaccine alone, whereas this increase does not exceed the 14 times in Balb/c mice, for the group treated with MF59 + CpG.

Fig. 13c shows that immunization with vaccine alone promotes no detectable levels of anti-H3N2 A IgG2a antibodies (indicative of Th1 response) in C57BL/6 mice. Furthermore, IgG2a levels are detected in Balb/c mice. In general, the quality of the humoral response is the same between Balb/c and C57BL/6 mice: there is a predominant type-2 response, dominated by high IgG1 antibody titres, induced by MF59 and a clear shift toward a type-1 response after CpG addition. Interestingly, in C57BL/6 mice immunized with MF59 adjuvanted vaccine the differences between IgG1 and IgG2a antibody production are less marked than in Balb/c mice. This is easier to observe in **Fig. 14**, where the ratios IgG2a:IgG1 antibody titres are lower in C57BL/6 mice treated with vaccine + MF59 than in Balb/c mice. Instead, by adding CpG to MF59 adjuvanted vaccine the IgG2a:IgG1 ratio is similar among the two mouse strains. Nevertheless, the major discrepancy between cellular and humoral responses appears to be at the level of C57BL/6 mice immunized with 0.3 μg/ml of Flu antigens adjuvanted with MF59 + CpG: the IFNγ:IL-5 ratio is clearly much higher than IgG2a:IgG1 ratio.

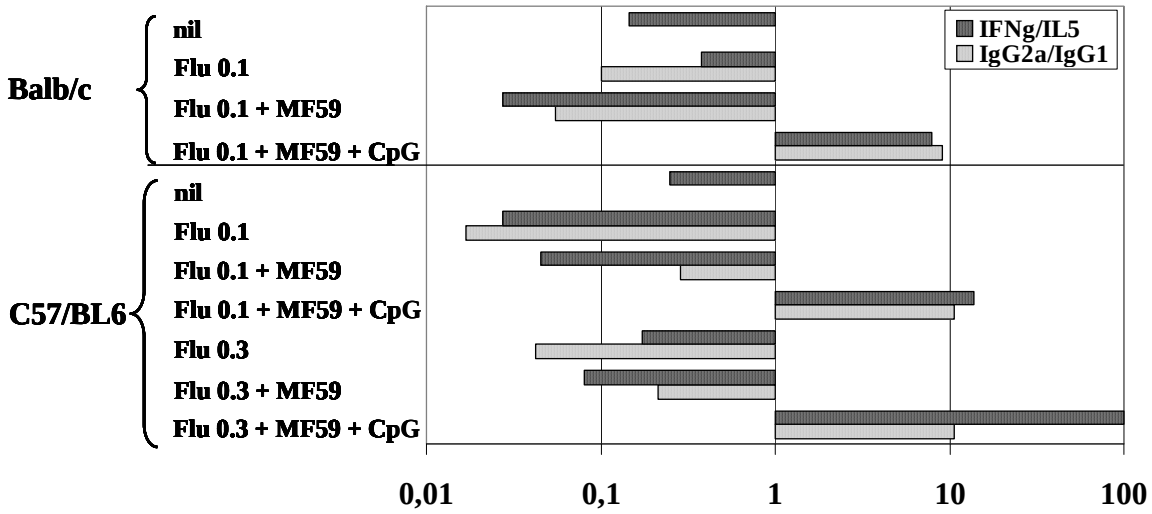


Fig. 14: The quality (type-1/type-2) of humoral and cellular response to Flu vaccine, alone or adjuvanted, is independent of genetic background of mouse strains used.
Balb/c mice were immunized intramuscularly twice, at weeks 0 and 3, with influenza vaccine (0.1 μg of each antigen derived from influenza strain H1N1 A/New Caledonia, H3N2 A/New York and B/Jiangsu) either alone (Flu) or adjuvanted as indicated. At 2 weeeks after 2nd immunization immunization mouse spleens and sera were taken and analyzed for T cell and antibody responses. Darker bars (▩) represent the ratios of IFNγ positive divided by IL-5 positive CD4+ T cells. Lighter bars (▨) represent the titre ratios of IgG2a:IgG1 isotype geometric mean titres against H3N2 A.

These results clearly show that the quality of immune responses to adjuvanted Flu vaccine is not influenced by genetic background of mice used in experiments: MF59 can be defined as a Th0 adjuvant, which enhances whichever response is present, and the combination of MF59 + CpG promotes a Th1 type adjuvantation. The only established difference that occurs by using different mouse strains concerns the magnitude of immune response.

4.7 Comparison between immune responses induced by immunization with adjuvanted vaccine and viral infection

Adjuvants offer the opportunity to drive immune responses to subunit vaccine towards a desired Th profile, by selecting the appropriate types and their combinations. We have previously demonstrated that MF59 adjuvanted vaccine promotes a Th2 immunity, and the addition of CpG to MF59 adjuvanted vaccine results in a strong Th1 immune response. Influenza virus infection normally induces a strong type-1 response [179-182], which results in the activation of Th1 response, secretion of IFNγ cytokine and production of high levels of IgG2a serum antibody [180].

To compare the efficacy of a Th2 vs. a Th1 promoting Flu vaccine, in this study we have investigated the effects of different vaccination regimens (inducing type-1 or type-2 responses) on immune response, in comparison with the influenza virus infection.

Different groups of Balb/c mice were either immunized intramuscularly twice, 3 weeks apart, with vaccine (0.2 μg of Ag derived from influenza strain H1N1 A/Solomon) alone, vaccine + MF59 and vaccine + MF59 + CpG, or alternatively intranasally infected with one or two sublethal doses (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon) of influenza virus. At 2 weeks after 2nd treatment mouse spleens and sera were collected and cellular and humoral responses were analyzed (the experimental model is shown in **Fig. 15**).

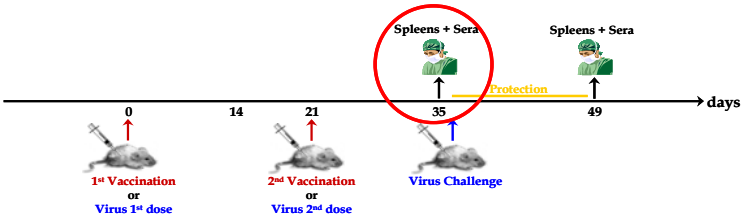


Fig. 15: The experimental outline.

A Th2-polarized immunity, dominated by IL-5 production, is obtained after 2 immunizations with vaccine alone and vaccine + MF59, whereas vaccine + MF59 + CpG generates a Th1-type response, with high IFNγ production, similar to the infection with one or two sublethal doses of influenza virus (**Fig. 16a**). Interestingly, T cell response after one infection is weak and lower than those induced by both types of adjuvanted

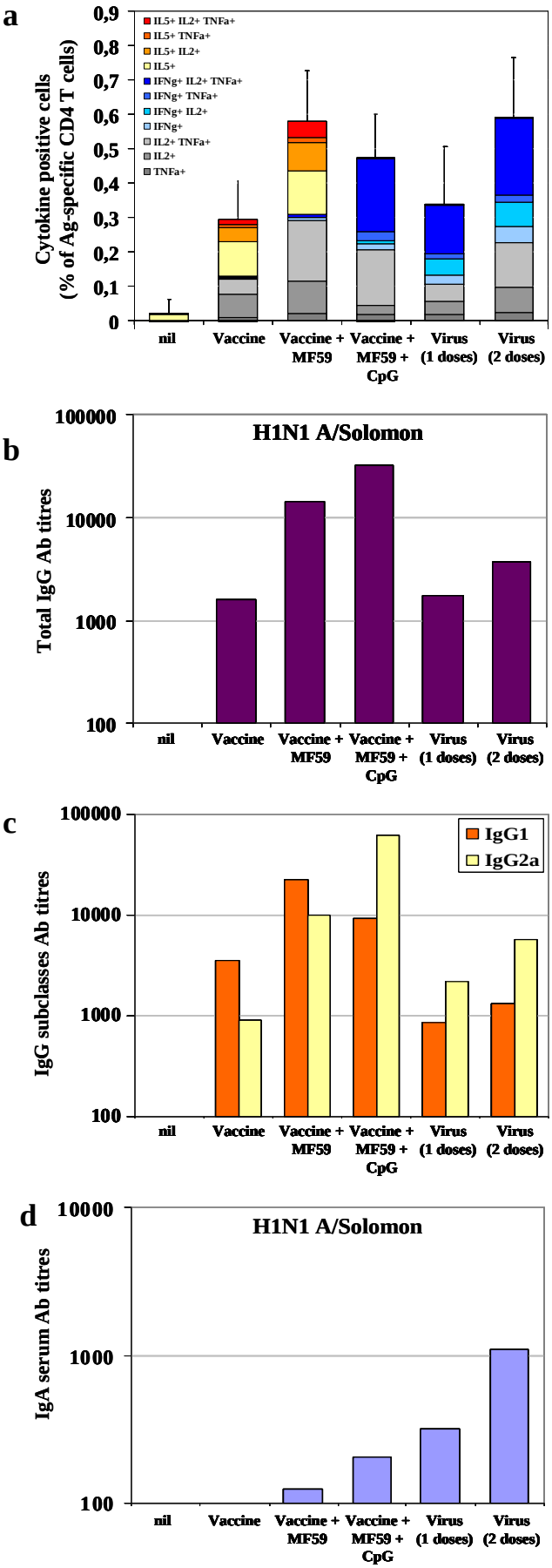


Fig. 16: Profiles (type-1 or type/2) of immune responses induced by Flu vaccines differently adjuvanted and influenza virus infection.

Balb/c mice were pre-exposed to 1 or 2 sublethal doses of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon) or immunized intramuscularly twice with Flu vaccine (0.2 µg of Ag derived from influenza strain H1N1 A/Solomon) either alone or adjuvanted as indicated. At 2 weeeks after 2nd immunization mouse spleens and sera were collected. Splenocytes were re-stimulated in vitro with the H1N1 A/Solomon antigen and intracellular cytokine staining was performed. Histograms in **Fig. 16a** show CD4+ T cell responses 2 weeks post 2nd immunization, extimated by the frequency of CD4+ T cells producing cytokines. Each bar represents the mean of the response of splenocytes from three spleens. Total IgG serum titres against H1N1 A/Solomon were determinated by ELISA and the geometric means of serum IgG titres against H1N1 A (**Fig. 16b**) 2 weeks post second dose (3 mice/group) are shown. **Fig. 16c** and **d** show respectively HA-specific IgG subclasses and IgA titres (geometric mean titres) against H1N1 A measured by ELISA on mouse sera 2 weeks post 2nd immunization.

vaccines, whereas 2 virus doses increase T cell response to levels comparable with the MF59 adjuvanted vaccine.

Adjuvants promote higher anti-HA total IgG titres than vaccine alone or exposure to one or two doses of virus (**Fig. 16b**). Moreover, when vaccines are formulated with MF59 + CpG total IgG titres are enhanced compared to MF59 adjuvanted vaccine, in contrast with data on T cell responses. Again, mice exposed to 2 doses of virus show a higher humoral response than mice exposed to virus only one time. Nevertheless, both groups of infected mice generate humoral responses slightly higher than mice immunized with vaccine alone. Total IgG antibody production is particularly low in mice exposed to one dose of virus, probably because this is the only group whose sera were collected 5 weeks after the treatment (see **Fig. 15**), while in all other cases sera were collected 2 weeks after the 2^{nd} treatment.

The type-2 immunity generated by vaccine alone or adjuvanted with MF59 and the type-1 profile of immune responses induced by vaccine + MF59 + CpG or exposure to virus are confirmed by the analysis of IgG isotypes production (**Fig. 16c**). MF59 adjuvanted vaccine promotes IgG1 antibody titres higher than IgG2a, whereas humoral responses in mice immunized with MF59 + CpG or infected with influenza virus are dominated by IgG2a production. Again, treatments with adjuvanted vaccines are more immunogenic than exposure to virus.

Adjuvanted subunit vaccines, inoculated intramuscularly, stimulate great anti-HA serum IgG antibody production. However, the same vaccine formulations induce only weak levels of serum IgA antibodies, which are important for protection against influenza virus infection (**Fig 16d**). Serum IgA titres were measured at 2 weeks post 2^{nd} treatment (except for mice exposed to one dose of virus, whose sera were collected 5 weeks post infection) by ELISA. IgA antibodies are not detected in mice immunized with vaccine alone. On the other hand, IgA titres generated by influenza virus, inoculated intranasally, are higher than those induced by all vaccine formulations; after 2 exposures to virus serum IgA titres increase up to 14-fold in respect to vaccine alone, 9-fold in respect to MF59 adjuvanted vaccine and 3.5-fold in respect to vaccine adjuvanted with MF59 + CpG.

Fig. 17 shows that the correlation between type-1 and type-2 profiles at humoral and cellular levels is maintained not only in immunized mice, but also in infected mice.

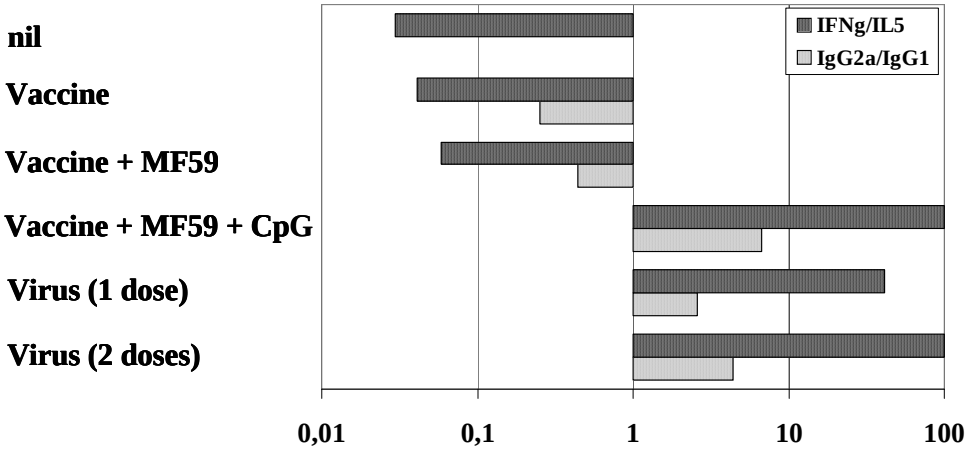


Fig. 17: Correspondence between cellular and humoral immune response profiles in both immunized and infected mice.
Balb/c mice were pre-exposed to 1 or 2 sublethal doses of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon) or immunized intramuscularly twice with Flu vaccine (0.2 μg of Ag derived from influenza strain H1N1 A/Solomon) either alone or adjuvanted as indicated. At 2 weeeks after 2^{nd} immunization immunization mouse spleens and sera were taken and analyzed for T cell and antibody responses. Darker bars (▩) represent the ratios of IFNγ positive divided by IL-5 positive CD4+ T cells. Lighter bars (▨) represent the titre ratios of IgG2a:IgG1 isotype geometric mean titres against H1N1 A.

4.8 Evaluation of neutralizing antibody titres induced by adjuvanted vaccines and exposure to virus

Adjuvanted vaccines induce higher anti-HA total IgG antibody titres than vaccine alone or exposure to virus. In order to determine whether the produced antibodies are able to neutralize virus, preventing viral entry and infection, the same sera analyzed in **Fig. 16b-d** were tested for the presence of neutralizing antibodies by a Microneutralization Assay. Serial dilutions of sera were pre-incubated with H1N1 A/Solomon influenza virus for 1hour. Thus, MDCK cells were added and left with virus for 22 hours (if sera contain neutralizing antibodies, they should prevent the viral entry into MDCK cells). Then, an ELISA was performed to detect the presence of viral nucleoprotein (NP) and determine the neutralizing activity, expressed as percentage of neutralization for each serum dilution (for more details see materials and methods).
Neutralizing assay shows that adjuvanted vaccines, regardless of the quality (type-1 or type-2) of immune response they induce, generate antibodies more effective in virus neutralization than vaccine alone and exposure to virus (**Fig 18a**). MF59 adjuvanted

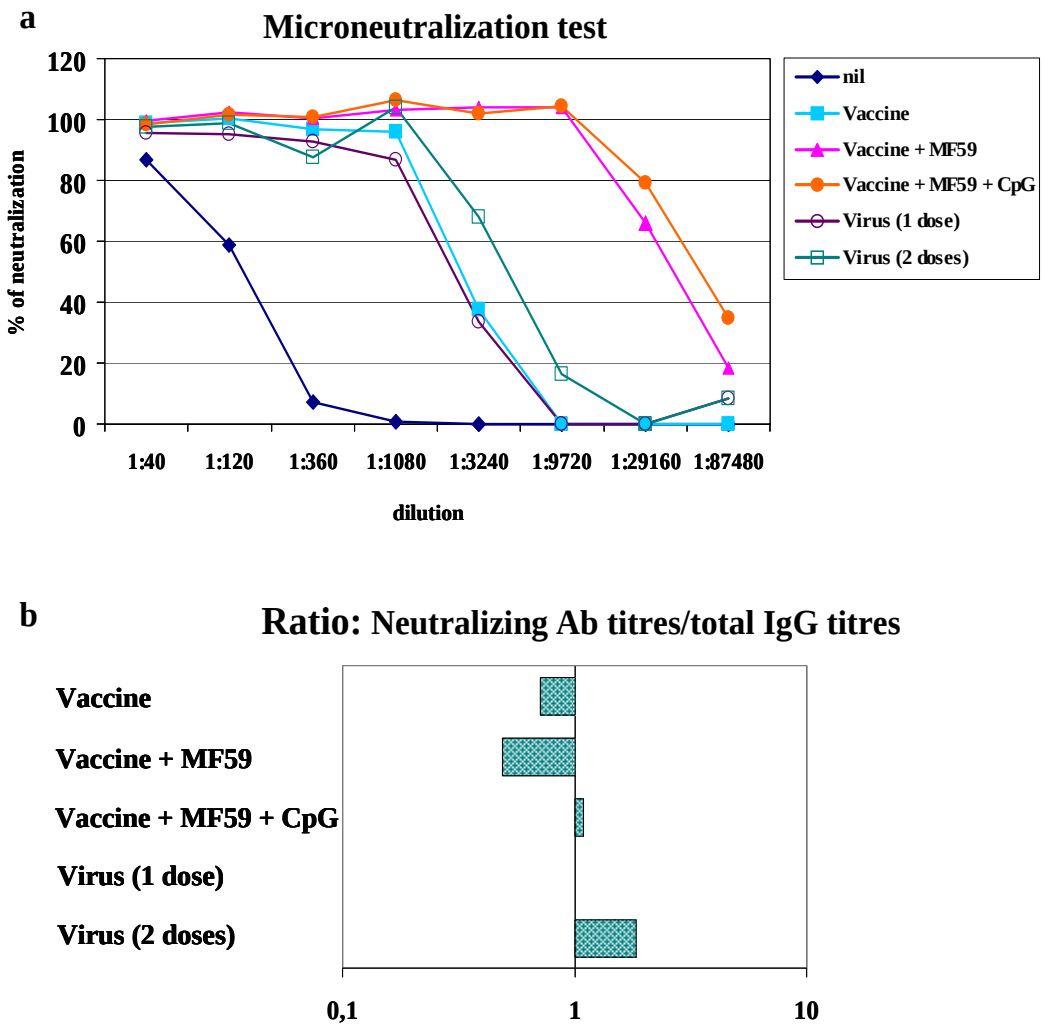


Fig. 18: Adjuvanted vaccines are the most potent treatments to induce effective neutralizing antibodies.
Balb/c mice were pre-exposed to 1 or 2 sublethal doses of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon) or immunized intramuscularly twice with Flu vaccine (0.2 µg of Ag derived from influenza strain H1N1 A/Solomon) either alone or adjuvanted as indicated. 2 weeks post second immunization pooled sera were heat inactivated, serially diluted and incubated with 300 $TCID_{50}$ of virus (influenza strain H1N1 A/Solomon) for 1 h at 37°C. 100 µl of MDCK cells at 1.5×10^5/ml was then added to each well. After 22 h at 37°C, the presence of viral protein was detected by ELISA with monoclonal antibodies to the influenza A NP and M proteins (**Fig. 18a**). The inhibitory concentration (IC_{80}) was calculated by interpolation and expressed as the reciprocal dilution of the sample giving an 80 % reduction in viral antigen. IC_{80} values correlate with serum neutralizing antibody titres.
Fig. 18b shows the ratios of IC_{80} values:total IgG titres against H1N1 A.

vaccine, which stimulates a Th2 response, and vaccine adjuvanted with MF59 + CpG, which stimulates a Th1 response, both induce serum antibodies that neutralize the virus up to high serum dilutions. Neutralizing antibody levels in mice immunized with vaccine alone or exposed to one dose of virus are comparable, and slightly lower then those in mice exposed to two sublethal doses of virus.

To determine if the serum total IgG levels induced by immunization or infection (**Fig. 16b**) correlate with the neutralizing activity of sera observed between the same treatments, we calculated the inhibitory concentration IC_{80} (expressed as the reciprocal dilution of the sample giving an 80% reduction of viral antigen), which correlates with serum neutralizing antibody titres. The ratios between IC_{80} values and total IgG antibodies were calculated and represented in **Fig. 18b**. The ratio between neutralizing antibody titres and total IgG titres is low in mice immunized twice with vaccine alone and vaccine + MF59. It is interesting to note that these treatments induce both a Th2 immune response. Otherwise, two immunizations with vaccine containing MF59 + CpG and two exposures to influenza virus determine higher ratios between neutralizing antibody titres and total IgG titres. Interestingly, again the two treatments induce the same type of immune response, i.e. Th1. These findings suggest that other humoral components could contribute to neutralizing activity of mouse sera (and not only IgG antibodies), in particular in mice exposed twice to virus. In contrast, 2 immunizations with vaccine alone or adjuvanted with MF59 induce antibodies less effective in viral neutralization.

4.9 Protection against lethal influenza virus infection in mice vaccinated and pre-exposed to virus

The results presented above show that adjuvanted vaccines induce strong cellular and antibody responses in mice after two immunizations. Based on these results, we further tested whether these mice would be protected against lethal challenge by influenza virus. To measure the protective efficacy of the various vaccination regimens (promoting type-1 or type-2 responses) vs. pre-exposure to influenza virus, 2 weeks after the 2^{nd} immunization/infection 6 mice per treatment, from experiment in **Fig. 16-18**, were challenged intranasally with a lethal dose (10 MLD_{50}) of homologous influenza virus. Mice were checked daily for survival and their weight loss was monitored for 2 weeks after lethal infection as correlate of protection (the experimental model is shown in **Fig. 19**).

The weight loss for mice of each treatment is plotted in **Fig. 20** as a percentage of the average weight before challenge versus days after challenge. Almost all of the control group mice succumb to challenge and die between days 4 and 11 after challenge. All the

other animals, both vaccinated and pre-exposed to virus, initially display a severe body weight reduction and then, after the 4th day after challenge, they start to regain weight and

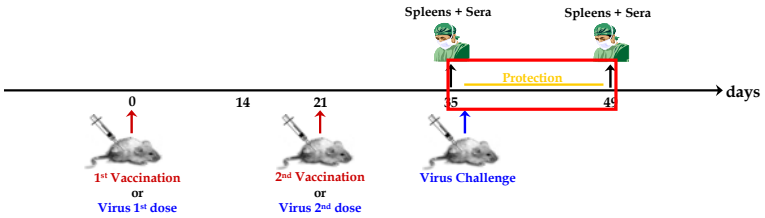


Fig. 19: Experimental challenge model.

survive. No significant differences in weight loss are observed after challenge for mice vaccinated with vaccine alone, vaccine + MF59, vaccine + MF59 + CpG and mice pre-exposed to one dose of virus. In contrast, mice pre-exposed to two sublethal doses of virus exhibit minor weight loss than the others, even if immunized mice with adjuvanted vaccines showed highest neutralizing antibody titres (**Fig. 18a**).

These results indicate that previously measured antibody response is not directly associated with protection from lethal influenza virus infection. In addition, the different types of immune response that we can induce by using different adjuvant combinations (type-1 or type-2) are both protective against viral infection. Thus, protection does not appear to be associated with a specific profile of immune response. In contrast, different degrees of protection are detected between immunized and pre-exposed mice, as in mice infected with two sublethal doses of virus weight loss after challenge is less severe than in the other groups.

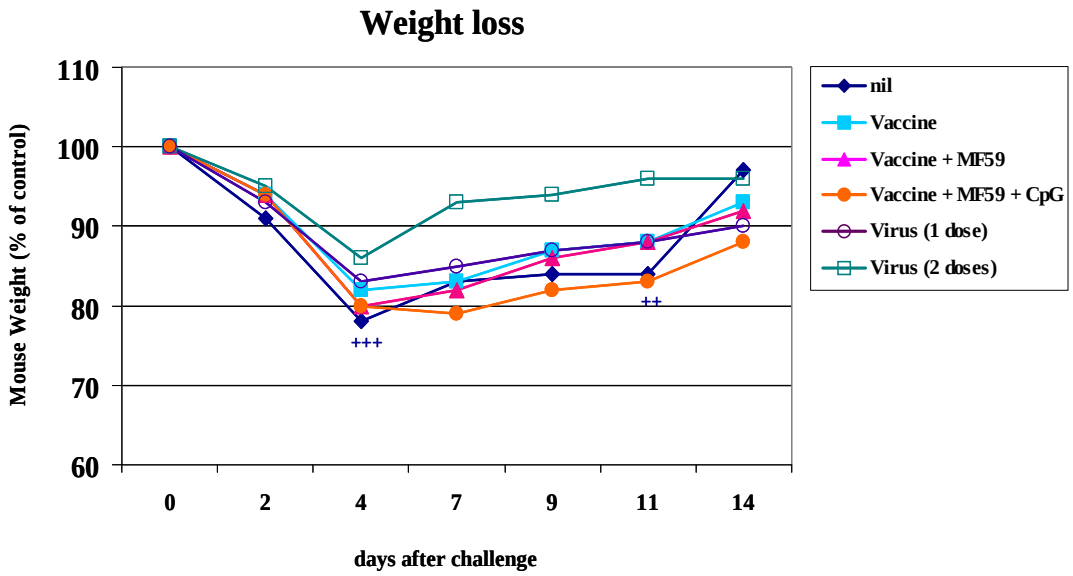


Fig. 20: Virus pre-exposure and vaccination confer protection against lethal challenge with homologous influenza virus.
Groups of 6 Balb/c mice were immunized intramuscularly twice with Flu vaccine (0.2 µg of Ag derived from influenza strain H1N1 A/Solomon) either alone or adjuvanted as indicated. 2 groups of mice were pre-exposed to 1 or 2 sublethal doses of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon). At week 5, mice were challenged intranasally with 10 MLD_{50} of homologous virus. Survival rates and average weight loss are shown.

4.10 Evaluation of cellular and humoral immune responses after challenge with homologous influenza virus

To investigate the profiles of immune response after challenge, survived mice were killed 2 weeks after lethal infection, mouse spleens and sera were collected and evaluated for cytokine and antibody production respectively.

As shown in **Fig. 21a**, the percentage of cytokine producing CD4 + T cells greatly increases in mice immunized with vaccine containing MF59 + CpG. Lethal challenge induces strong cellular response also in mice pre-exposed to one or two sublethal doses of virus, but at lower levels than in mice immunized with MF59 + CpG. Surprisingly, in mice treated with vaccine alone or formulated with MF59, the profile of cellular response remains clearly Th2 dominated by IL-5 production even after challenge, although viral infection promotes a Th1-type immune response, as shown by IFNγ production in challenged mice previously treated with PBS. These data suggest that homologous challenge does not change the profiles of pre-established immune responses, maintaining

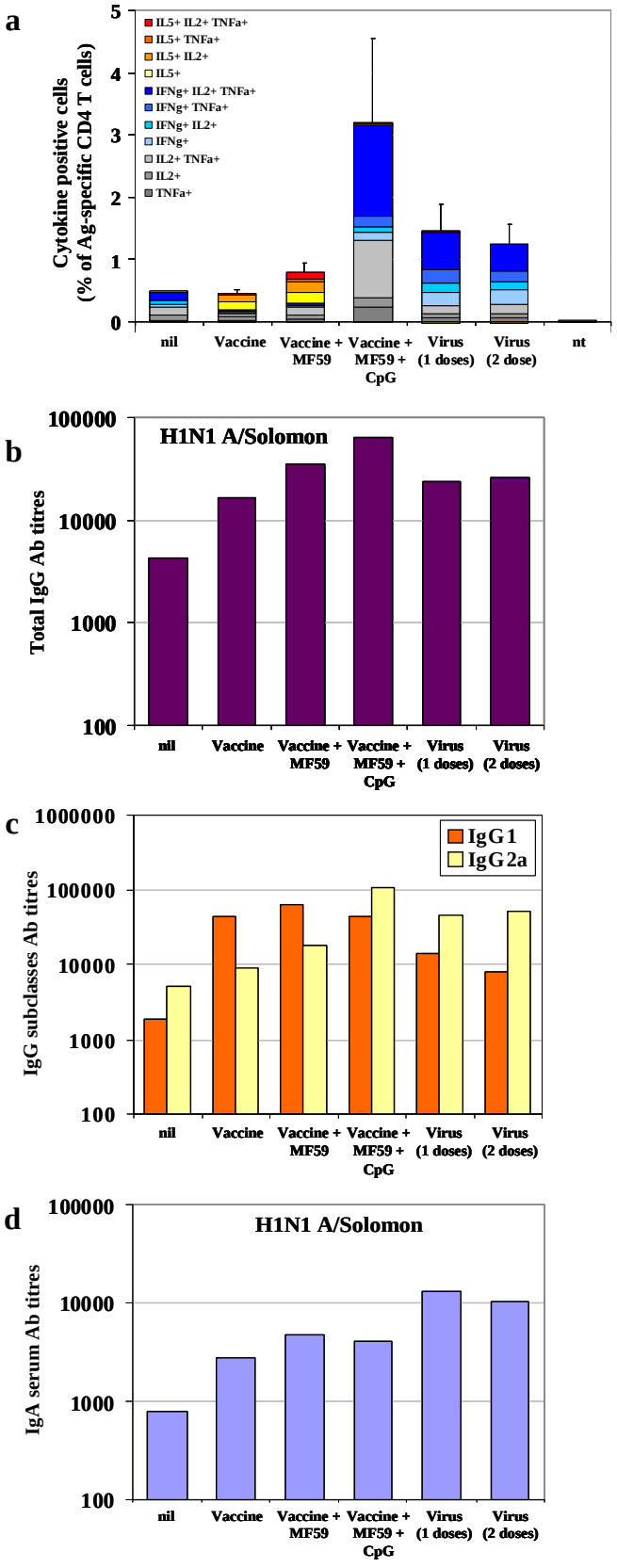


Fig. 21: The quality of humoral and cellular immune responses (Th1/Th2) doesn't change after homologous lethal challenge.

Balb/c mice were pre-exposed to 1 or 2 sublethal doses of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon) or immunized intramuscularly twice with Flu vaccine (0.2 μg of Ag derived from influenza strain H1N1 A/Solomon) either alone or adjuvanted as indicated. At week 5, mice were challenged intranasally with 10 MLD_{50} of homologous virus. 2 weeeks post challenge spleens and sera from recovered mice were collected. Splenocytes were re-stimulated in vitro with the H1N1 A/Solomon antigen and intracellular cytokine staining was performed. Histograms in **Fig. 21a** show CD4+ T cell responses, extimated by the frequency of CD4+ T cells producing cytokines. Each bar represents the mean of the response of splenocytes from three spleens. Total IgG serum titres against H1N1 A/Solomon were determinated by ELISA and the geometric means of serum IgG titres against H1N1 A (**Fig. 21b**) 2 weeks post challenge (3 mice/group) are shown. **Fig. 21c** and **d** show respectively HA-specific IgG subclasses and IgA titres (geometric mean titres) against H1N1 A measured by ELISA on mouse sera 2 weeks post challenge.

the observed skewed responses towards Th2 for vaccine and MF59 adjuvanted vaccine, and Th1 for MF59 adjuvanted vaccine + CpG and pre-exposure to virus.

Also serum antibody response was investigated after lethal challenge, measuring HA-specific total IgG (**Fig. 21b**), IgG subclasses (**Fig. 21c**) and IgA (**Fig. 21d**) antibody titres. The overall magnitude of total IgG antibody production is considerably increased compared to post 2nd vaccination/infection (**Fig. 16b**), as expected considering the strength of the viral stimulus. Again, the most potent responses are observed when mice were immunized with adjuvanted vaccines, in particular with vaccine adjuvanted with MF59 + CpG. No differences in total IgG levels are found in mice pre-exposed to one or two sublethal doses of virus (**Fig. 21b**).

Interestingly, the data obtained by analyzing IgG1 and IgG2a antibody production (**Fig. 21c**), confirm that the type-1/type-2 polarization of immune response established prior to challenge is maintained also after challenge. High titres of the IgG2a antibody isotype, which denote type-1 immunity, are measured in mice pre-exposed to virus and immunized with vaccine + MF59 + CpG after challenge. IgG1 titres are less than IgG2a in the same sera. Conversely, IgG1 titres remain higher than IgG2a titres in mice vaccinated with vaccine alone and MF59 adjuvanted vaccine, even after challenge. Again, mice treated with PBS before challenge show a type-1 humoral response after challenge, dominated by IgG2a production.

As expected, also IgA titres are greatly increased following viral challenge (**Fig. 21d**). Unlike IgG titres that are highly produced in mice immunized with adjuvanted vaccines (**Fig. 21b**), the highest IgA titres are measured in mice pre-exposed to virus, in agreement with what observed in **Fig. 16d**, post 2nd immunization/infection. In addition, after challenge IgA antibody levels are similar between mice pre-exposed to one and two sublethal doses of virus, whereas before challenge IgA titres generated by two doses of virus were appreciably higher than those induced after one dose.

To summarize the data on humoral and cellular response after viral challenge, the ratios between antibody titres IgG2a:IgG1 and the frequency of IFNγ:IL-5 producing T cells were calculated and represented in **Fig. 22**. A type-1 response at both the cellular and humoral levels is clearly visible also in pre-infected mice and mice immunized with vaccine containing MF59 + CpG. The challenge induces type-1 response, characterized by IFNγ and IgG2a production, in not vaccinated mice. Nevertheless, challenge does not influence the type-2 profile of immunity induced by vaccine alone and MF59 adjuvanted

vaccine. In fact, the profiles of immune responses induced by immunized and infected mice are similar to the pre-challenge data (**Fig. 17**).

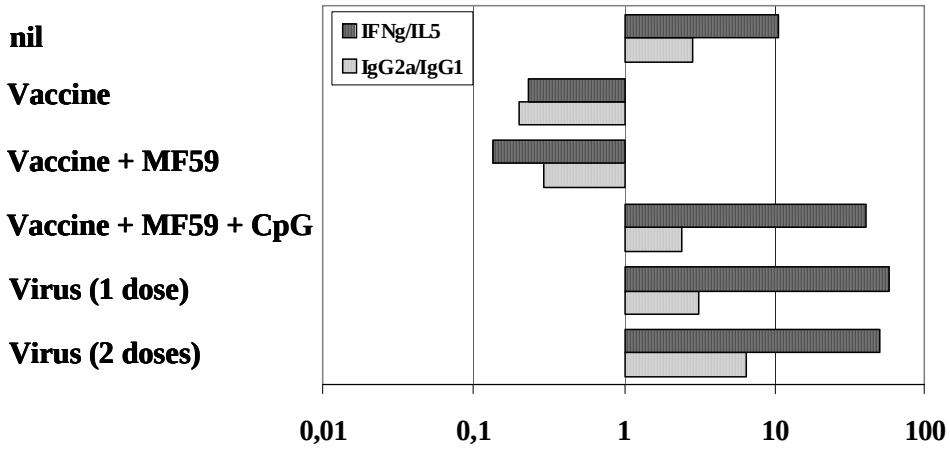


Fig. 22: Homologous challenge does not influence the type-1/type-2 ratios of both cellular and humoral immune responses.
Balb/c mice were pre-exposed to 1 or 2 sublethal doses of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon) or immunized intramuscularly twice with Flu vaccine (0.2 μg of Ag derived from influenza strain H1N1 A/Solomon) either alone or adjuvanted as indicated. At week 5, mice were challenged intranasally with 10 MLD_{50} of homologous virus. 2 weeeks post challenge spleens and sera from recovered mice were collected and analyzed for T cell and antibody responses. Darker bars (▩) represent the ratios of IFNγ positive divided by IL-5 positive CD4+ T cells, whereas lighter bars (□) represent the titre ratios of IgG2a:IgG1 isotype geometric mean titres against H1N1 A.

Also neutralizing antibodies were evaluated in survived mice 2 weeks after challenge (**Fig. 23**). Challenge greatly increases neutralizing antibody titres in all mice. The lowest antibody titres are measured in mice neither immunized nor pre-exposed to virus (nil), whereas no substantial differences are detected in the other groups after challenge.

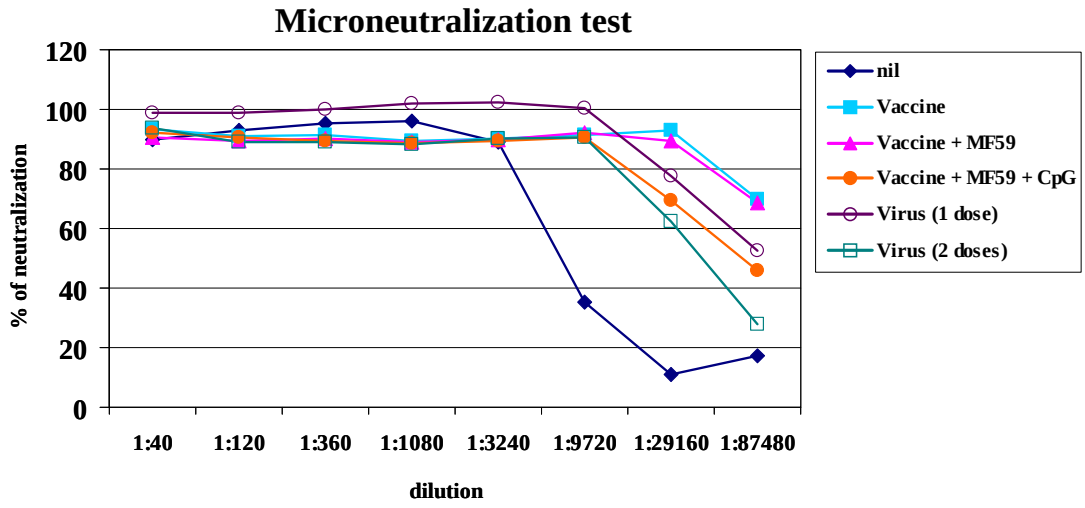


Fig. 23: Neutralizing antibody titres after homologous challenge.
Balb/c mice were pre-exposed to 1 or 2 sublethal doses of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon) or immunized intramuscularly twice with Flu vaccine (0.2 µg of Ag derived from influenza strain H1N1 A/Solomon) either alone or adjuvanted as indicated. At week 5, mice were challenged intranasally with 10 MLD_{50} of homologous virus. 2 weeks post challenge pooled sera from recovered mice were heat inactivated, serially diluted and incubated with 300 $TCID_{50}$ of virus (influenza strain H1N1 A/Solomon) for 1 h at 37°C. 100 µl of MDCK cells at 1.5×10^5/ml was then added to each well. After 22 h at 37°C, the presence of viral protein was detected by ELISA with monoclonal antibodies to the influenza A NP and M proteins.

4.11 Passive immunization of naïve mice with immune sera provides protection against lethal challenge

Our data showed that adjuvants enhance both cellular and humoral immune responses to subunit influenza vaccine and drive them into a desired direction (Th1 vs. Th2), by choosing the appropriate combinations. After two immunizations we detected strong CD4+ T cell responses and measured high serum antibody levels by ELISA and Microneutralization assay. We also found that immunity induced by adjuvanted vaccines is protective against lethal challenge with homologous influenza virus. Given that antibodies are a crucial component of protective immunity to infection, we decided to investigate more in detail the contribution of serum antibodies, and more generally of humoral immunity, in mediating protection against lethal influenza virus infection. For this purpose, we performed a passive transfer experiment, in which immune sera from mice vaccinated or exposed to virus were transferred into naïve recipients, subsequently challenged with a lethal dose of homologous influenza virus. Immune sera were collected 2 weeks after 2^{nd} immunization or 5 weeks after exposure to virus, administrated intra-

abdominally in naïve mice, and 24 hours later mice were challenged with 3 MLD_{50} of influenza virus (experimental model shown in **Fig. 24**).

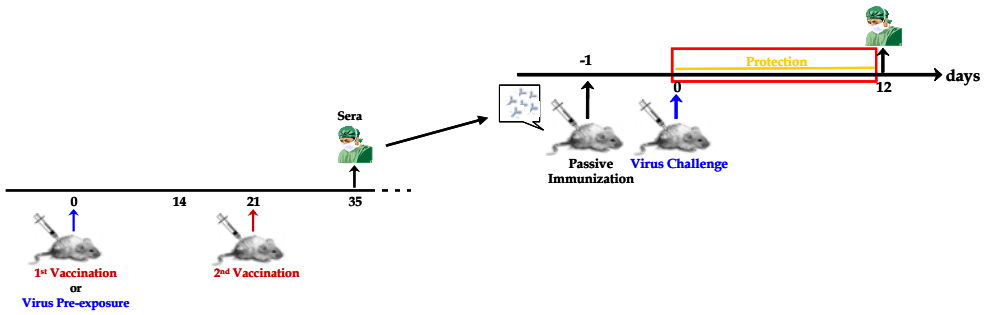


Fig. 24: Passive immunization model.

Survival was followed for 2 weeks and average weight loss is represented in **Fig. 25**.

All the animals, except control mice represented with the green line, show body weight reduction between days 5 and 7 after challenge.

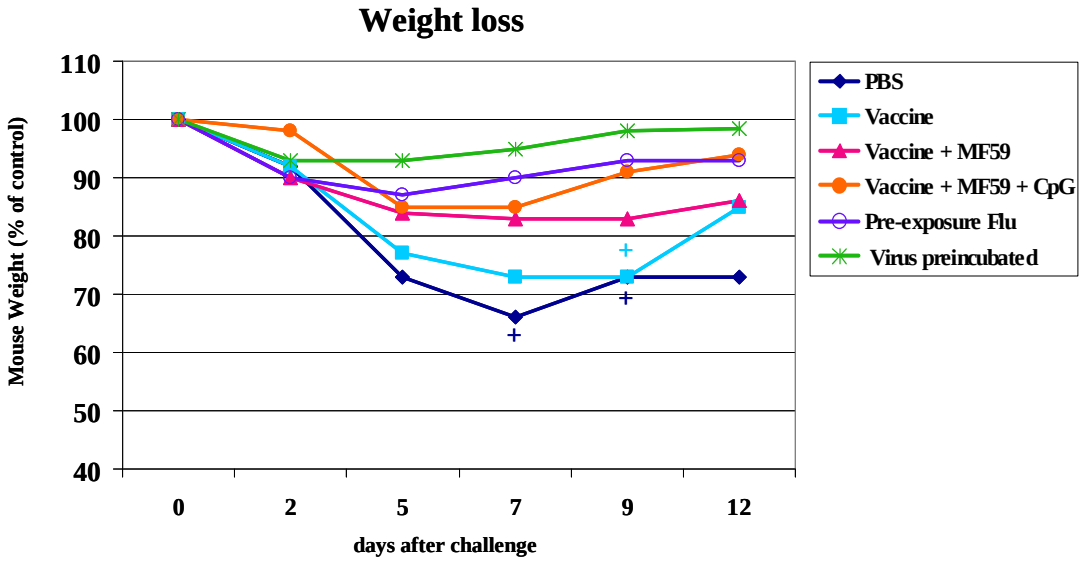


Fig. 25: Transfer of immunized sera into naïve mice confers protection against lethal challenge with homologous influenza virus.
Balb/c mice were pre-exposed to a sublethal dose of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon) or immunized intramuscularly twice with Flu vaccine (0.2 µg of Ag derived from influenza strain H1N1 A/Solomon) either alone or adjuvanted. Pooled sera from mice vaccinated or infected as indicated were administrated intra-abdominally to naive mice. 24 hours later, mice were challenged intranasally with 3 MLD_{50} of homologous virus. In the group (✳) before challenge virus was pre-incubated with sera from mice pre-exposed to virus. Survival and average weight loss were followed for two weeks.

In the control group before challenge virus was pre-incubated with sera from mice pre-exposed to virus, containing neutralizing antibodies. Passive transfer of sera from mice administered only with PBS can not protect recipient mice from a lethal influenza infection: 2/6 mice die between days 7 and 9 after challenge, and the 4 survivors do not recover even after 14 days. Also the sera from mice immunized twice with vaccine alone are not very protective, with one mouse found dead after 9 days and the others showing a weight loss of almost 30% (often the established endpoint in mouse challenge experiments is a body weight reduction of 30% with respect to initial weight, when the mice are killed to prevent further suffering). Conversely, sera from mice immunized twice with adjuvanted vaccines or exposed to one sublethal dose of virus confer protection to naïve mice against an otherwise lethal infection with homologous influenza virus. All the mice receiving these three kinds of sera show an initial body weight reduction 5 days after challenge. Then, mice passively immunized with sera from MF59 adjuvanted vaccine do not regain weight in 2 weeks, instead mice receiving sera either from mice immunized twice with vaccine containing MF59 + CpG or from mice exposed to virus recover after day 5 and show increased body weights. Thus, our data suggest that humoral components generated in mice in which is induced a type-1 immunity (by pre-exposure or immunization with vaccine + MF59 + CpG) are more effective in protection from lethal homologous challenge than those generated in mice vaccinated with MF59 adjuvanted vaccine, which promotes a type-2 immunity.

4.12 Influence of pre-exposure to influenza virus on immune response to Flu vaccine

Unlike humans, who are annually exposed to the circulating strains of influenza virus and in which, normally, Flu-specific immunity is already present at the time of vaccination, mice used in our experiments are naïve to exposure to influenza virus. Consequently, we tried to improve the predictability of the mouse model by evaluating subunit Flu vaccine in mice that had previously been infected with influenza virus, to understand if, and eventually how, previous Flu infection influences the outcome of vaccination. For this purpose, we performed an experiment in which three groups of Balb/c mice were primed with PBS, a sublethal dose of influenza virus (10^3 $TCID_{50}$ of influenza strain H1N1

A/Solomon) or vaccine (0.2 μg of Ag derived from influenza strain H1N1 A/Solomon) + MF59. 3 weeks after the first treatment each group was further divided in 3 subgroups and boosted with vaccine alone, vaccine + MF59 or PBS. Subsequently, at 2 weeks after boost mouse spleens and sera were taken and analyzed respectively for CD4+ T cell and antibody responses (experimental model shown in **Fig. 26**).

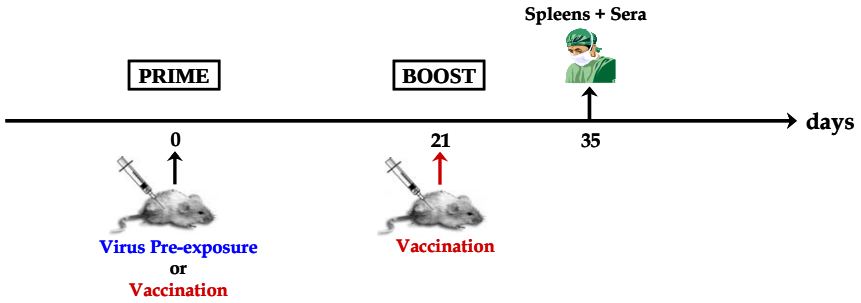


Fig. 26: Experimental model of mouse pre-infection.

Fig. 27 shows CD4+ T cell cytokine production induced in splenocytes restimulated in vitro with antigens from different influenza virus strains: H1N1 A/Solomon (**Fig. 27a**), H1N1 A/Brisbane (**Fig. 27b**) and H3N2 A/Brisbane (**Fig. 27c**).

This was done to investigate the heterologous response eventually induced by vaccine formulations. In fact, since the induction of heterosubtypic immunity to influenza viral antigens is of paramount importance for the prevention of epidemics and potential pandemics, the development of improved vaccines, which will be effective also against viral strains different from those included in the vaccine is an ongoing goal of vaccinologists.

Data show that immunizations with vaccine alone or adjuvanted with MF59 induce a Th1 response, dominated by IFNγ production, in mice previously exposed to a sublethal dose of influenza virus, whereas in mice unprimed or primed with MF59 adjuvanted vaccine promote Th2 immunity (confirming the findings of prior experiments) (**Fig. 27a-c**). Thus, pre-exposure to virus influences the outcome of immune response to vaccine, by biasing it toward a Th1 profile which is maintained also after immunization with MF59 adjuvanted vaccine.

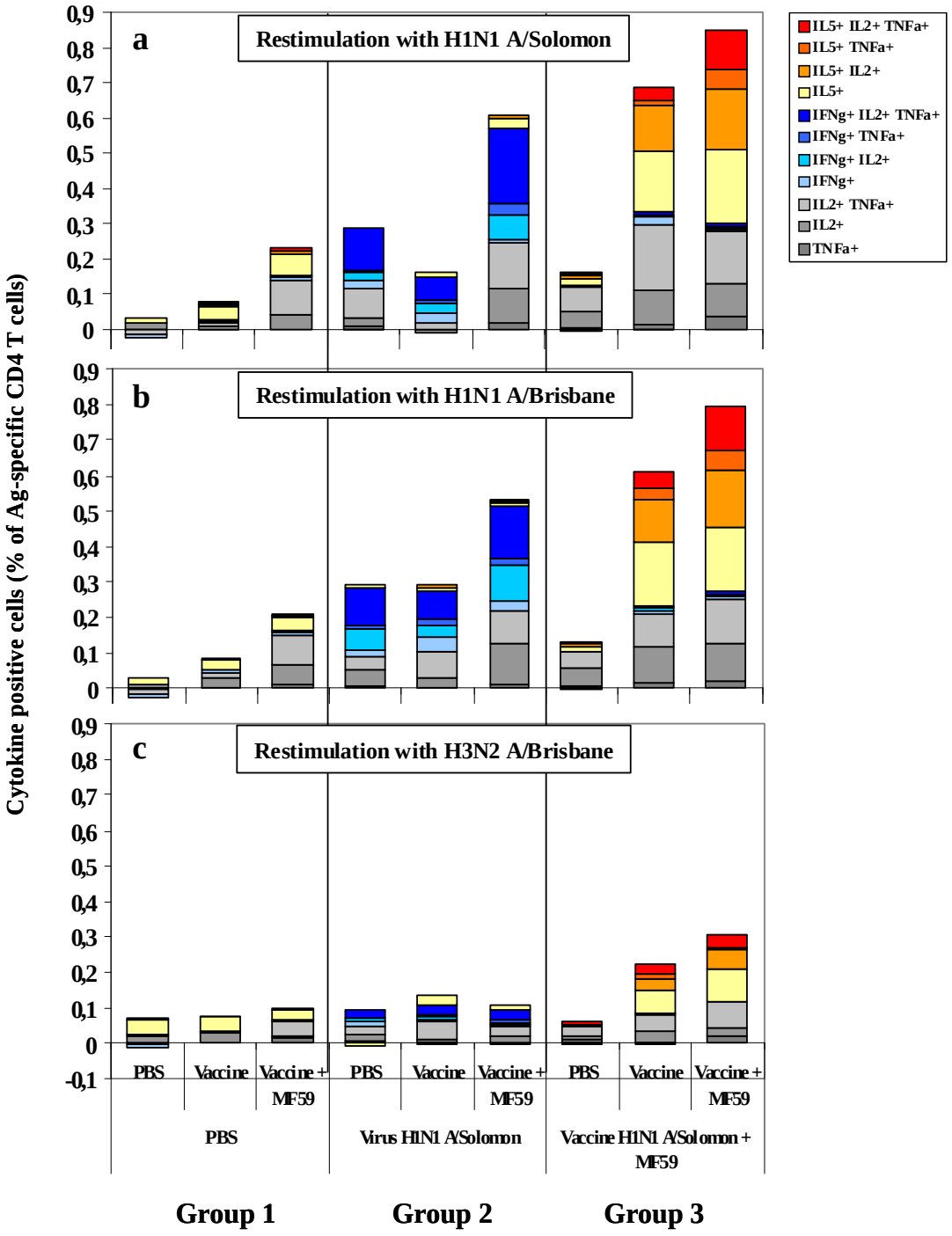


Fig. 27: Virus pre-exposure influences the outcome of vaccination: CD4+ T cell response to Flu antigens.

Balb/c mice were divided in three groups: group 1 was treated with PBS; group 2 was pre-exposed to a sublethal dose of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon); group 3 was immunized with Flu vaccine (0.2 μg of Ag derived from influenza strain H1N1 A/Solomon) + MF59. 3 weeks after treatments each group was further divided and immunized with vaccine, vaccine + MF59 or PBS. At 2 weeks after 2nd immunization mouse spleens were collected and splenocytes were in vitro restimulated with antigens from different influenza strains, as indicated. Histograms show CD4+ T cell responses 2 weeks post 2nd immunization, extimated by the frequency of CD4+ T cells producing cytokines, to antigens H1N1 A/Solomon (**Fig. 27a**), H1N1 A/Brisbane (**Fig. 27b**) and H3N2 A/Brisbane (**Fig. 27c**). Each bar represents the response of splenocytes from three pooled spleens.

Interestingly, no differences in terms of quality and magnitude of cellular responses, are found between cells restimulated in vitro with antigens from H1N1 A/Solomon (the same strain used for infection and immunization) and with antigens from H1N1 A/Brisbane (a heterovariant strain, serologically distinct from H1N1 A/Solomon, even if belonging to the same subtype)(**Fig. 27a-b**). Although there is a great similarity between the two mouse strains, these data indicate that vaccine formulations can induce a cross-reactive T cell response to new drifted variant strains. In contrast, cells from the same groups of mice do not respond to stimulation with antigens from H3N2 A/Brisbane (a heterosubtypic strain, strongly dissimilar from the other two) in terms of cytokine production (**Fig. 27c**). The overall magnitude of T cell response promoted by vaccine alone or adjuvanted is higher in mice primed with vaccine + MF59 than in mice pre-exposed to virus, with an evident adjuvant effect in both cases (**Fig. 27a-b**). Adjuvanted and unadjuvanted vaccines promote a very low cellular response in mice primed with PBS when splenocytes are stimulated in vitro with H1N1 A/Solomon and H1N1 A/Brisbane; this response is very low compared to our usual experiments, even if we consider that mice were immunized only one time. Conversely, pre-exposure to virus induce a strong Th1 response in mice then administered with PBS, even higher than that promoted by a subsequent immunization with Flu vaccine (**Fig. 27a**).

Similar qualitative results are obtained by the analysis of HA-specific IgG1 and IgG2a antibody titres against H1N1 A/Solomon (**Fig. 28**). Priming with MF59 adjuvanted vaccine (group 3) induces an antibody response clearly dominated by IgG1 subclass, indicative of a Th2-type response. Also one dose of vaccine + MF59 stimulates IgG1 production, whereas IgG isotypes promoted by vaccine alone are undetectable (group 1). Regarding the profile of humoral responses in mice pre-exposed to virus (group 2), however, it is clearly Th1 type, dominated by IgG2a isotype, when mice were subsequently administered with PBS or vaccine alone. Surprisingly, the immunization with MF59 adjuvanted vaccine after exposure to virus, in addition to increasing antibody production, promotes the shift of humoral response to vaccine from a IgG2a- to a IgG1-dominated isotype (typ-2 biased). In fact, immunization with vaccine + MF59 following viral infection induces a type-1 cellular response, with high IFNγ production with respect to IL-5 (**Fig. 27a-b**), but a more balanced humoral response, shifted toward a type-2 profile, with IgG1 titres higher than IgG2a (**Fig. 28**).

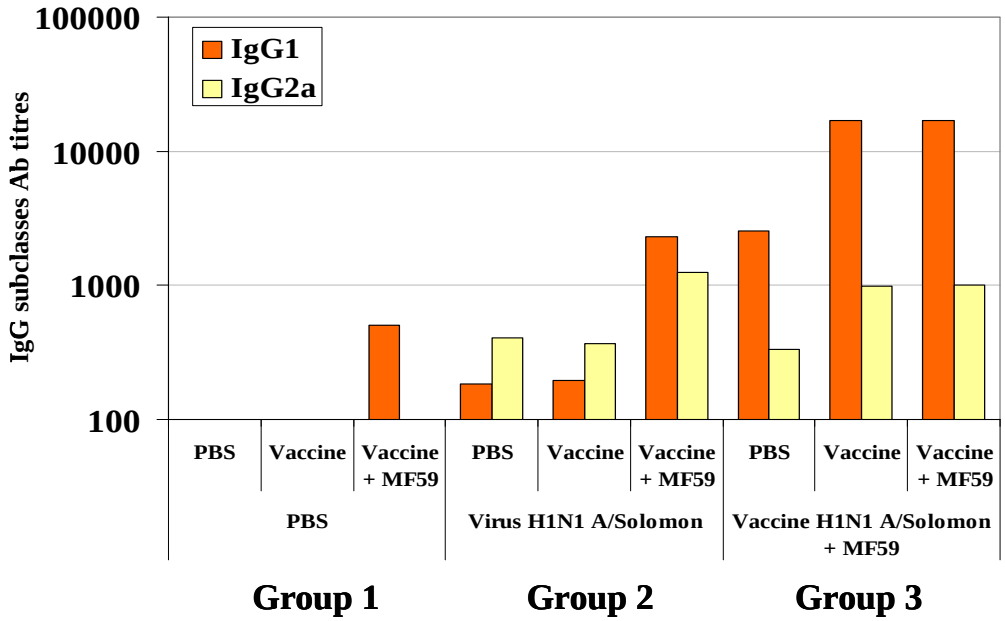


Fig. 28: Virus pre-exposure influences the outcome of vaccination: IgG subclasses antibody titres. Balb/c mice were divided in three groups: group 1 was treated with PBS; group 2 was pre-exposed to a sublethal dose of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon); group 3 was immunized with Flu vaccine (0.2 μg of Ag derived from influenza strain H1N1 A/Solomon) + MF59. 3 weeks after treatments each group was further divided and immunized with vaccine, vaccine + MF59 or PBS. At 2 weeks after 2nd immunization mouse sera were collected. Figure shows HA-specific IgG1 and IgG2a titres (geometric mean titres) against H1N1 A/Solomon measured by ELISA on mouse sera 2 weeks post 2nd immunization.

Thus, the correspondence between cytokines and antibody isotype is lost in this case and this is well visible in **Fig. 29**, where the ratios between IgG2a:IgG1 antibody isotype and IFNγ:IL-5 cytokine production are shown. In all other cases the profiles (typ-1/type-2) of cellular and humoral immune responses correlate and there is an evident bias of immune response toward a type-1 or type-2 profile. In addition, in mice exposed to virus the shift of humoral response toward a type-1 profile is not so evident, with IgG2a:IgG1 ratios lower than IFNγ:IL-5 ratios. This indicates that the humoral response is qualitatively more balanced than the cellular one.

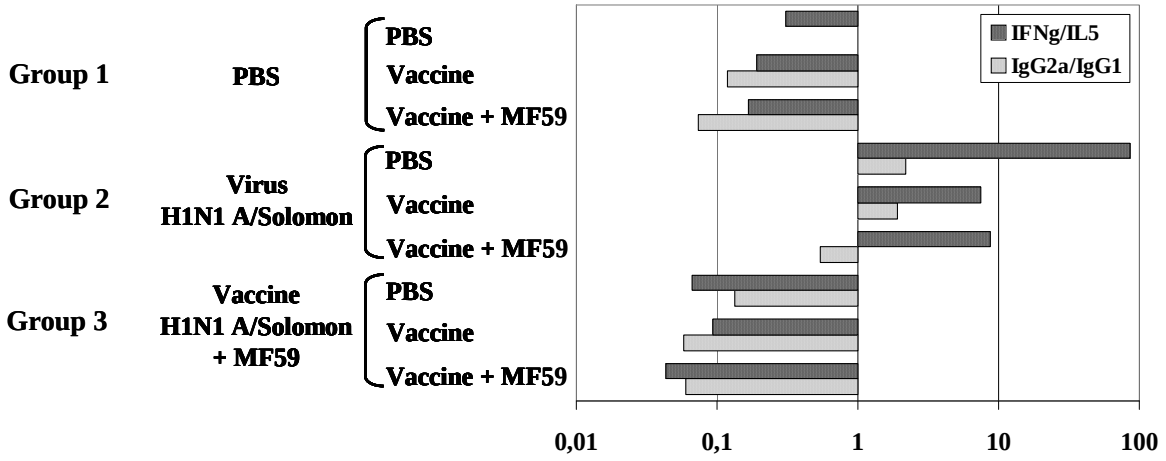


Fig. 29: Pre-exposure to virus reduces the correlation between cellular and humoral immune response to influenza vaccines.
Balb/c mice were divided in three groups: group 1 was treated with PBS; group 2 was pre-exposed to a sublethal dose of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon); group 3 was immunized with Flu vaccine (0.2 μg of Ag derived from influenza strain H1N1 A/Solomon) + MF59. 3 weeks after treatments each group was further divided and immunized with vaccine, vaccine + MF59 or PBS. At 2 weeks after 2nd immunization mouse spleens and sera were collected and analyzed for T cell and antibody responses. Darker bars (▩) represent the ratios of IFNγ positive divided by IL-5 positive CD4+ T cells, whereas lighter bars (▨) represent the titre ratios of IgG2a:IgG1 isotype geometric mean titres against H1N1 A.

We also checked for the presence of neutralizing antibodies on mouse sera that were collected 2 weeks following immunization after prime with virus or adjuvanted vaccine (**Fig. 30**).

It has been shown previously (**Fig. 18a**) that exposure to virus induces antibodies less effective in virus neutralization than adjuvanted vaccine. Here we confirmed the above finding, as priming with vaccine + MF59 generate higher neutralizing antibody titres than pre-exposure to influenza virus also in mice then administrated with PBS. However, the sera from mice vaccinated with a single dose of subunit vaccine show no neutralizing properties. Similar neutralizing activity is found in sera from mice only exposed to virus and mice pre-exposed to virus and then immunized with vaccine alone. Instead, when pre-exposed mice are subsequently immunized with vaccine + MF59, neutralizing antibody titres in the sera increase, indicating that it clearly depends on the MF59 adjuvant. The highest neutralizing antibody titres are measured in mice primed with MF59 adjuvanted vaccine and then boosted. Interestingly, the neutralizing activity of sera does not change if the 2nd immunization was done with vaccine alone or adjuvanted with MF59.

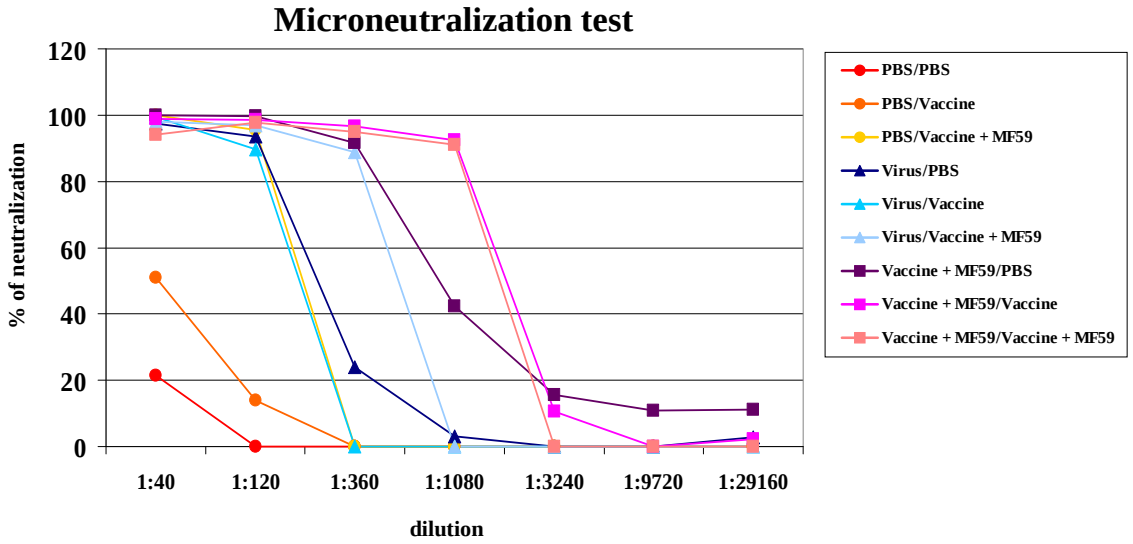


Fig. 30: 2 doses of Flu vaccine induce more effective neutralizing antibodies than exposure to virus before vaccination.
Balb/c mice were divided in three groups: group 1 was treated with PBS; group 2 was pre-exposed to a sublethal dose of virus (10^3 $TCID_{50}$ of influenza strain H1N1 A/Solomon); group 3 was immunized with Flu vaccine (0.2 µg of Ag derived from influenza strain H1N1 A/Solomon) + MF59. 3 weeks after treatments each group was further divided and immunized with vaccine, vaccine + MF59 or PBS. At 2 weeks after 2nd immunization pooled sera were heat inactivated, serially diluted and incubated with 300 $TCID_{50}$ of virus (influenza strain H1N1 A/Solomon) for 1 h at 37°C. 100 µl of MDCK cells at 1.5×10^5/ml was then added to each well. After 22 h at 37°C, the presence of viral protein was detected by ELISA with monoclonal antibodies to the influenza A NP and M proteins.

Thus, the adjuvant effect of MF59 in improving the efficacy of Flu vaccine has been demonstrated again. We also demonstrated that pre-exposure to virus increases neutralizing antibody titres in respect to mice not pre-exposed. However, we found that 2 immunizations with vaccine adjuvanted with MF59 induce a more potent immune response than pre-exposure to virus followed by single immunization.

To summarize, the comparisons among mice differently primed (with Flu virus, Flu vaccine adjuvanted with MF59 or PBS) and then vaccinated suggest that pre-exposure to virus, which mimics human experience, influences the outcome of vaccination. Consequently, to improve the predictability of mouse model, we should use mice previously exposed to virus. Interestingly, the ability of MF59 adjuvant to enhance cellular and humoral immune responses to Flu vaccine is confirmed also in pre-exposed mice.

5. DISCUSSION

Influenza virus continues to be a major cause of morbidity and mortality worldwide despite the availability of vaccines and antiviral agents. Vaccines containing highly purified subunit antigens by themselves are safe but generally weakly immunogenic, especially in the populations most at risk (infants, elderly and immunocompromised individuals). One strategy for improving current influenza vaccines is to use them in conjunction with adjuvants to increase their immunogenicity. This concept is not new, but most adjuvants tested thus far have either failed to provide the needed effect or are too toxic for routine use in humans. Recently, a number of adjuvants for influenza vaccine were compared in mice, showing that MF59 significantly outperforms various alternatives, for both antibody and T cell responses [137].

Growing consideration of the importance of cell-mediated (Th1) immunity in the protection against intracellular pathogens like influenza virus has substantiated the benefit from an immune response beyond antibody production and B cell memory in order to prevent disease [183]. This, together with a better understanding of the immune system, especially regarding the impact of innate and adaptive immunity and their close interaction, has allowed for a more rational approach in the design of new vaccines including the use of adjuvants. For almost one century, Alum has been the only vaccine adjuvant approved for use in humans worldwide. Only in the last decade other additional adjuvants, such as the oil-in-water emulsion MF59 and the TLR4 agonist monophosphoryl lipid A (MPL) formulated in Alum (AS04), have been licensed by the European Medicinal Evaluation Agency [184]. MPL adjuvant has been used extensively in clinical trials, which demonstrated the efficacy of an attenuated TLR4 agonist as a

vaccine adjuvant. Presently, two TLR4 agonist containing vaccines are approved for use in humans, namely Fendrix® for the prevention of hepatitis B and the cervical cancer vaccine Cervarix™ [146-148, 185 and 186]. In both cases MPL is formulated with a delivery system. Furthermore adjuvant systems have also been tested in various influenza vaccine programmes [116]. Novel lipid A mimetics that lack a disaccharide backbone, nevertheless retaining TLR4 stimulatory activity were recently described by Eisai [149-151]. One of these synthetic compounds, E6020, was found to be more potent than MPL, but preclinically safe at the dosing levels required for vaccine adjuvanticity [151]. Recently, it has been developed the two-component synthetic adjuvant IC31, signaling through TLR9, with characteristics that are likely to contribute to improving influenza vaccines. IC31 is a mixture of a novel immunostimulatory oligodeoxynucleotide (ODN1a) and the peptide $KLKL_5KLK$. Studies revealed that it strongly stimulates both T and B cell responses with type 1 dominance when combined with different types of antigens [139, 187 and 188], without induction of undesirable side effects. This indicates its potential for novel vaccine formulations.

In the current study the MF59 adjuvant is compared with a range of alternative adjuvants, including the new generation immune-potentiators CpG, E6020 and IC31. While MF59 induced a potent Th2 immune response dominated by IL-5 producing CD4+ T cells and the IgG1 antibody isotype, we showed that the addition of CpG to MF59 adjuvanted vaccine promotes a shift of the immune response from a type-2 to a type-1 profile, which results in the secretion of IFNγ cytokine and production of high levels of IgG2a serum antibodies. The adjuvant effects of CpG on the immune response to Flu vaccine were demonstrated to be dose-dependent, because the type-1 profile of immune response induced by CpG addition to MF59 adjuvanted vaccine switches back to a Th2-type by lowering the CpG dosage. When MF59 is co-delivered whit E6020 or IC31 immune-potentiators, the shift of immune responses toward a Th1-type is less evident than in the case of CpG, with the generation of a more balanced type-1/type-2 immunity (especially with IC31). MF59 adjuvant and its combination with CpG seemed to induce potent long-term immune responses to subunit influenza vaccine that resulted in a sustained antigen-specific CD4+ T cell response even 4 months after 2^{nd} immunization. In addition, we showed that the quality of immune response (Th1 or Th2) promoted by different vaccination regimens is not influenced by the genetic background of mouse strain used in the experiments, finding the same profiles of cellular responses between Th1-prone and

Th2-prone mouse strains. Further investigations on the protective efficacy of Flu vaccine adjuvanted with MF59 alone or MF59 + CpG demonstrated that, although both types of adjuvanted vaccines induce levels of neutralizing antibody titres higher than those generated by exposure to influenza virus, pre-exposed mice exhibit minor weight loss than mice immunized with adjuvanted vaccines when challenged with a lethal dose of virus. Finally, we found that, in contrast to naïve mice, mice pre-exposed to influenza virus show a clear Th1 response to MF59, suggesting that the pre-established immune status influences the outcome of vaccination.

Previous studies have shown that MF59 is a surprisingly potent stimulator of both humoral and cellular immune responses against a variety of alternative antigens [161], and also that it possesses a favorable safety profile. The significant potency of MF59 observed in our studies is in line with previous data and has important implications for the development of optimal Flu vaccines against inter-pandemic strains and in preparations for an influenza pandemic. Also in the context of pediatric CMV and HIV vaccines, MF59 was shown to be a well tolerated, potent adjuvant [189 and 190]. Hence, MF59 has significant potential for use as a broad range vaccine adjuvant in human vaccines [161] for a wide range of individuals of different ages.

Although MF59 is a more potent adjuvant when compared to CpG, E6020 or IC31 (this study), and other adjuvants like Alum, calcium phosphate and the delivery system poly-(lactide co-glycolide) as shown recently [137], it is mostly effective at enhancing antibody and T-cell proliferative responses [160 and 169], but it is not a powerful adjuvant for the induction of Th1 cellular immune responses, confirming results obtained in various preclinical models [160, 169 and 189]. Since the influenza virus induces Th1 responses and IFNγ and TNFα have been shown to have some antiviral effects [191], it may be desirable to induce a Th1 response against influenza and in other viral infections.

In Balb/C mice under SPF conditions, immune responses promoted by MF59 adjuvanted Flu vaccine are dominated by IL-5 producing CD4+ T cells and high levels of IgG1 antibodies, which indicate a Th2 polarization. The addition of CpG to MF59 allowed the induction of a potent Th1 response, characterized by the production of IFNγ and of IFNγ-dependent antibody isotype IgG2a. This finding is in line with previous ones showing that when combined with conventional vaccines, CpG shifts immune responses towards Th1 [137]. Also the combination of MF59 with E6020 and IC31 immune-potentiators results in a shift of the immune responses from a Th2- toward a more pronounced Th1-type, but

in these cases the shift is less marked, maintaining a more balanced immunity. In particular, we found that the addition of IC31 to MF59 induces both IFNγ and IL-5 producing T cells, which represents a mixed Th1/Th2 cytokine profile. In contrast with previous studies showing the induction of a strong type-1 cellular response by IC31 [139], we demonstrate that its addition to Flu vaccine promotes a balanced type-1/type-2 cellular response. Also a mixed type-1/type-2 humoral response is obtained in mice that received Flu vaccine adjuvanted with MF59 + IC31, in which the IgG2a antibody levels increase remarkably and less profoundly those of IgG1. ODN1a, one component of IC31, is a TLR9 agonist signaling through MyD88 as CpG, which is clearly established to induce a strong Th1 response. Instead, ODN1a does not activate the immune system by itself, most probably due to its short half-life based on its phosphodiester backbone. The second component of IC31, $KLKL_5KLK$ (KLK), was recently described to induce type 2 immunity against the model protein OVA following a prime-boost vaccination approach [140]. The adjuvant properties of KLK are based on the induction of a depot formation at the injection site and on the enhanced uptake of antigens into APCs. The combination of the two components in IC31 forms a stable complex via ionic and hydrophobic interactions, in which the nuclease-sensitive ODN1a (which stimulates a type-1 response) and the antigens are protected against degradation. This is probably the reason why the interaction of these compounds leads, under the chosen experimental conditions, to potent immune responses of mixed type-1/type-2 characteristics, whereas CpG DNA activates exclusively type-1 immunity. Additionally, the long-lasting adjuvant effect of IC31 has been reported, which is most likely related to the already described induction of a depot of the IC31-containing vaccine formulations at the injection site [141].

The induction of a long-lasting, protective immune memory that is able to respond quickly to future infections and that could be rapidly boosted following a single dose of the vaccine is the goal of vaccination. Stimulated by this notion, we analyzed the long-term effects of MF59 and/or CpG on vaccine-induced immunity by measuring the IFNγ and IL-5 producing CD4+ T cells responses four months after 2 immunizations. Even at this late time-point, MF59 mediated an increase in the Th2-polarized immunity induced by subunit influenza vaccine and in the Th1-polarized immunity generated by CpG adjuvanted vaccine, although to a lower extent than observed 2 weeks after 2^{nd} immunization. These results show that the MF59 adjuvant can increase the quantity and duration of the immune response by promoting the generation of large and long-lasting

pools of memory T cells. The addition of immunomodulatory molecules, such as TLR9 agonist CpG, can influence the functional profile of the memory cells. Therefore, by choosing the appropriate adjuvants or their combinations we are able to induce potent, polarized and sustained cellular responses and this is particularly important for the development of vaccines against potential pandemic influenza virus strains.

For influenza the anti-viral role of interferons is well established, and experiments *in vitro* show anti-viral activity of IFNγ as well as TNFα against human influenza viruses on lung epithelial cells [192]. Given the synergistic antiviral effect of IFNγ and TNFα, it is an important observation that a high proportion of T cells induced by MF59 + CpG, MF59 + E6020 or MF59 + IC31 produce both these cytokines. In addition, IL-2 is a central autocrine T cell growth factor important for the maintenance of T cells and therefore, for memory. In influenza infections cytokine responses are known to be involved in the early and critical stages of host defense [193]. More generally, the induction of multi-cytokine producing T cells through vaccination has been associated with increased protection [98, 194-196], and the majority of IFNγ producing CD4+ cells found here also secrete IL-2 and TNFα thus responding to the requirement of inducing multifunctional cells as suggested in these studies.

The finding that the co-delivery of MF59 adjuvant with Flu vaccine formulated with CpG, E6020 or IC31 immune-potentiators did not modify the quality of the immune response but significantly amplified both IgG1 and IgG2a isotype antibody titres, while maintaining the ratio IgG2a:IgG1 unchanged, leads to the conclusion that MF59 can be more precisely defined as a neutral adjuvant, which enhances whichever response is present, without biasing the profile. In other more Th1 prone experimental settings, such as in mice pre-exposed to influenza virus, MF59 simply increased the magnitude of the pre-existing Th1 response, further indicating that MF59 enhances immune responses in an essentially neutral manner. This ‘‘neutrality’’ of MF59 may make it an ideal vehicle to deliver adjuvants, which have the potential to strongly bias the immune response.
Recent studies [197 and 198] revealed that MF59 enhances the immune response at a range of points, including the induction of chemokines to increase recruitment of immune cells to the injection site, enhanced antigen uptake by monocytes at the injection site and enhanced differentiation of monocytes into DCs, important for priming naive T cells. An important feature of MF59 is that it facilitates the migration of DCs into draining lymph

nodes where they can trigger the adaptive immune response specific to the vaccine [197 and 198]. On the other hand, the direct activation effects on DCs is very bland compared to that by immunostimulants such as CpG or LPS, which might be the reason for the lack of bias imposed on the ensuing T cell and antibody response and thus may explain why MF59 is a rather neutral adjuvant with respect to Th1–Th2 bias. In contrast, it is conceivable that E6020 (which engages the TLR4), CpG and IC31 (both engaging the TLR9) activate DCs directly to produce inflammatory cytokines, such as IL-12 or IFNα and other factors promoting Th1 induction.

There may be considerable differences in the type of immune responses to foreign antigens in various mouse strains. To exclude the possibility that the profiles of immune responses could depend on the genetic background of mouse strain used to perform the experiments (Balb/c mice are Th2-prone), we included in our investigations additional studies on C57BL/6 mice (more type 1 prone), confirming the mouse strain-independent immunostimulatory properties of MF59 adjuvanted vaccine. In fact, subunit Flu vaccine + MF59 was found to generate high levels of IgG1 antibodies and IL-5 producing CD4+ T cells in both C57BL/6 and Balb/c mice. In addition, the co-delivery of MF59 and CpG leads to a shift of immune response towards a Th1 type in both mouse strains of different genetic background.

Interestingly, we found that the induction of a strong shift of immune response from a Th2- to a Th1-type when CpG is co-delivered with MF59 is a dose-dependent effect of the TLR9 agonist. The IFNγ dominated response decreases by lowering the CpG dose, switching back to a IL-5 dominated response at the lowest concentrations of CpG, and also the IgG2a:IgG1 ratios decrease in a dose-dependent manner. The magnitude of the cellular response to vaccine adjuvanted with MF59 + CpG is only weakly affected by the variations in the CpG concentration. Similarly, the humoral response (total IgG antibody production) seems to reach a plateau after 2 immunizations with MF59 and no variation in the total IgG titres is observed at different CpG doses. Thus, altering the ratio between MF59 and the immune-potentiator may allow to control and direct the quality of the immune response induced by the vaccine formulation, without affecting its magnitude. This finding provides crucial information for further investigations and has important implications in the vaccine design.

As individual adjuvant, MF59 induced optimal cellular and humoral responses, whereas none of the tested immune-potentiators (CpG, E6020 or IC31) when administered as single adjuvant with the influenza vaccine were able to induce high CD4+ T cell or antibody responses as MF59 does. On the other hand, MF59 was not able to induce effective Th1 responses, which could be achieved by the addition of an immune-potentiator. Importantly, we demonstrated that the co-delivery of MF59 with some TLR agonists promotes the generation of a strong immune response to subunit influenza vaccine, opening the possibility to drive and modulate its quality into a desired direction.

To address in depth which type of immune response (type-1 or type-2) is most efficient at protecting from influenza infection, we analyzed the type-2 response obtained with MF59 adjuvanted vaccine and the type-1 response obtained with vaccine adjuvanted with MF59 + CpG, in comparison with the type-1 immunity induced by influenza virus infection, which is known to be protective. Both types of adjuvanted vaccines generate highest levels of virus-specific antibodies (total IgG, IgG1 and IgG2a), which are very effective in virus neutralization (as tested by a Microneutralization Assay). Although the major antibody isotype present in the sera of mice that survive viral infections is IgG2a, our findings demonstrate that the different IgG subclasses induced by vaccination (IgG1 or IgG2a) play both an important role in the neutralization ability of immune sera, according to previous results which supported a role also for IgG1 antibodies in the neutralization of viral particles both *in vitro* and *in vivo* [199].

We also found that neutralizing antibody titres induced by adjuvanted vaccines were higher than that induced by the vaccine alone and the exposure to low virus doses. The conventional assay for the evaluation of vaccine-induced antibody response is the haemagglutination-inhibition (HI) assay. An alternative to the HI assay that might be more sensitive, providing more functional measure of vaccine-induced immunity, is the microneutralization assay [200]. Antibody levels as measured by both the HI assay and the microneutralization assay correlate with protection against clinical illness from influenza infection [201]. Thus, our data seem to suggest that immunity induced by both types of adjuvanted vaccine, regardless of the profile (type-1/type-2) of the immune response that they promote, is more protective than that induced after viral infection. However, both types of immune sera from mice immunized with adjuvanted vaccines and from mice exposed to virus confer protection against lethal challenge when transferred into naïve mice. The ability of antibodies to neutralize influenza virus *in vitro* does not correlate perfectly with the protection of mice against lethal influenza challenge, thus

neutralization assays are not always predictive of *in vivo* resistance to viral infection from previous vaccination or exposure. A lack of correlation between *in vitro* and *in vivo* functions of antibodies to herpes simplex virus (HSV), Sindbis virus, vesicular stomatis virus, mumps virus and Semliki Forest virus has also been described [202]. The *in vivo* protective capacity of sera from exposed mice was underestimated by the *in vitro* neutralizing titer or capacity assays. It may be attributed to direct viral neutralization as well as antibodies interacting with other components of the immune system *in vivo*, such as complement, phagocytic cells, and natural killer cells. This combination of activities serves to synergize viral clearance via both humoral and cellular immune mechanisms. Thus, the *in vivo* effect of antibody depends on not only the antigen-binding variable region (F_{ab}), but also the constant region (F_c) that interacts with complement and cellular F_c receptors [203]. In fact, *in vitro* neutralization is mediated via direct binding of hypervariable region of antibodies to viral capsid proteins and sequestration, induction of viral aggregation or interference with viral attachment to cellular receptors [204]. *In vivo*, in addition to antibody-mediated direct inhibition of viral infectivity, viral particles are also cleared via opsonization. By coating viral particles with antibodies that also bind to F_c receptors on macrophages and neutrophils, antibodies stimulate enhanced phagocytosis and clearance of viral particles in vivo [205 and 206]. The complement-dependent lysis of infected cells bound with both neutralizing and non-neutralizing antibodies as a possible mechanism of antibody protection has already been described for other viruses [207-209]. The lysis of virus-infected cells by antibody-dependent cellular cytotoxicity (ADCC) may be another mechanism by which IgG antibodies afford protection, particularly for poorly neutralizing antibodies [210]. Indeed, this mechanism has been described for HSV [211 and 212].

The results presented here show not only that the humoral components in sera from pre-infected mice confer protection against influenza infection, but also that mice passively immunized with those sera recover more easily and rapidly than mice that received sera from donors immunized with MF59 adjuvanted vaccine. Our data on passive immunization experiments suggest that the type-1 humoral immunity, especially that induced by pre-exposure to virus, is more effective in protection from lethal challenge than the type-2 humoral response generated by MF59 adjuvanted vaccine. Current influenza vaccines are designed to elicit neutralizing antibody responses to the external molecules HA and NA. In contrast, natural infection with influenza virus elicits antibodies specific also for viral proteins different from that used in the vaccine formulations (i.e. NP

[213]), which may play a role in the control of viral infection. In addition, proteins separated from virus particles (as the vaccine proteins) are generally much less immunogenic than the intact particles. This difference in activity is usually attributed to the change in configuration of a protein when it is released from the structural requirements of the virus particle. Up to now, only limited informations are available on the effects of structural changes of the vaccine proteins on the immune response to influenza virus, but the disruption of intact virions and further purification of envelope glycoproteins for a subunit vaccine may induce conformational changes which modify the immunogenicity of the viral antigens. Furthermore, vaccine proteins exhibit more sites accessible to antibodies than those embedded in the intact viral envelope, allowing the production of a number of antibodies that could be ineffective in protection against viral infection, as they recognize epitopes that are masked on the intact virion. All these considerations are confirmed by our data showing that the ratio between neutralizing antibody titres and total IgG titres are higher in mice exposed to virus than in immunized mice. In fact, this suggests that viral infection leads to the activation of additional humoral components with respect to vaccination, which are effective in viral neutralization.

However, when mice immunized with adjuvanted vaccines and mice exposed to virus were directly infected with a lethal dose of influenza virus, they survived to challenge and both types of immune responses (type-1/type-2) induced by different adjuvant combinations are similarly protective against challenge. In contrast to the data from the passive immunization experiments, here protection does not appear to correlate with a specific profile of immune response. In contrast, different degrees of protection are detected between immunized and pre-exposed mice, with the pre-infected mice exhibiting minor weight loss than the others. The ability of infected mice to resist lethal challenge may result from the activation of components of the immune system that are not induced by adjuvanted vaccines. Indeed, the immune response to influenza viruses is also characterized by the production of IgA antibodies [64] and the activation of a potent CD8+ T cell (CTL) response [63].

Although systemically (intramuscularly) administered vaccines are known to induce serum IgG antibodies, they are generally poor stimulators of secretory IgA at respiratory mucosal sites [214-216]. In contrast, natural infection with influenza virus (intranasal infection) induces high levels of mucosal IgA antibodies. Many murine and human studies support the importance of mucosal IgA responses in protection against influenza

infection and disease [217] and, consequently, support also our data on infected vs vaccinated mice. Interestingly, the intranasal administration of vaccines has been shown to elicit an immune response directly at the mucosal level, increasing the production of secretory IgA antibodies. Therefore, recent efforts have focused on intra-nasal immunization strategies that induce both local IgA and systemic IgG responses [218-220]. As secretory IgA antibodies provide the first immunological barrier to pathogens that infect the epithelial surface, including influenza, and help to prevent the pathogen's invasion of deeper tissues, mucosal vaccination may have the potential to break the chain of infection at the earliest opportunity.

Also CD8+ T cells play a critical role in the influenza virus clearance and a number of different studies demonstrated that Flu infection leads to the activation of a strong virus-specific CD8+ T cell response. However, the induction of CD8+ T cell responses through vaccination, especially with current seasonal subunit vaccines, is clearly more difficult than inducing antibody or even CD4+ T cell responses. This could be the reason why we observed that infected mice are protected against challenge better than those vaccinated, even if adjuvanted vaccines induce high antibody titres (IgG and neutralizing antibodies) and antigen-specific CD4+ T cell cytokine production. In addition, virus-specific CD8+ T lymphocytes are very important in mediating heterosubtypic immunity (immune responses against heterovariant viral strains) [181].

Although the commonly used inactivated vaccines induce protective responses against the immunizing virus strains in a high proportion of the population [221], their effectiveness progressively declines as circulating viruses accumulate mutations in response to immune pressure. Vaccine strains are updated regularly but the ability to achieve a match with circulating strains is hampered by the lack of reliable methods for predicting future influenza virus evolution and the long lead time between the selection of vaccine strains and release of the vaccine. Moreover, irrespective of how closely they match circulating human strains, such vaccines are unlikely to provide significant protection against a novel subtype.

The continuing influenza epizootic in poultry and wild birds caused by the highly pathogenic A (H5N1) subtype, together with recent human infections by this and other avian type A influenza viruses, has stimulated interest in the development of vaccines that confer heterosubtypic immunity, with broad-spectrum of protection. Such cross-protective immunity is induced to some extent during natural infection, mediated mainly by CD8+ cytotoxic T lymphocytes that recognize epitopes located on the conserved internal

proteins of the virus as well as on the surface antigens [103, 222-224]. Unfortunately conventional inactivated vaccines do not induce CTL and only weakly boost existing memory CD8+ T-cell responses [225].

Modification of existing seasonal influenza vaccines to include a component that activates cross-protective T cells would offer an attractive strategy for improving community protection against drift variants of seasonal influenza viruses, at the same time reducing the impact of future pandemic strains. Strictly speaking, CD8+ T cells do not mediate protection against infection; they cannot exert their effector functions unless infection of target cells has occurred and their recognition structures on epithelial cells, MHC class I molecules bearing viral peptides, are generated. Rather, CTL act by limiting progression of disease to its most severe form, which, depending on the virulence of the virus strain, can include death and this is accomplished by lysis of infected cells before release of progeny virus. The resulting reduced viral load is more readily neutralized by the developing antibody response. Thus, the challenge for future influenza vaccines is to extend the spectrum of immunity induced to include heterosubtypic cross-protection.

Interestingly, although we didn't analyze the CTL responses to the vaccine formulations used in our experiments, we observed that MF59 adjuvanted vaccine induces cross-reactive antigen-specific CD4+ T cells that produce high levels of IL-5 after *in vitro* restimulation either with the same virus strain used in the vaccine (H1N1 A/Solomon) or with a heterovariant strain (H1N1 A/Brisbane). Additional investigations are needed to determine if adjuvanted vaccines (with MF59 adjuvant or adjuvant combinations promoting type-1 immunity) should be able to induce cross-reactive humoral and cellular responses and eventually if these confer protection against challenge with heterovariant strains. Furthermore, we found that CD4+ T cells are cross-reactive against H1N1 A/Brisbane strain, which is serologically distinct from the vaccine strain, but has great similarity with H1N1 A/Solomon, belonging to the same subtype. In contrast, no heterosubtypic activity was observed when cells were stimulated *in vitro* with the heterologous strain H3N2 A/Brisbane, which is strongly dissimilar from the vaccine strain. Therefore, further studies should improve the vaccine formulations also to extend their protective capacity against heterologous, unrelated virus strains. The role of CD4+ T cells in the heterosubtypic immunity has been studied less extensively than CD8+ T cells. Adoptive transfer of CD4+ T cells into nude mice accelerates neutralizing antibody responses to heterologous virus compared to wild-type mice [226], indicating that CD4+ T cell memory could potentially accelerate antibody levels during a heterosubtypic

infection. It has also been demonstrated that the depletion of CD4+ T cells prior to a subsequent challenge with a heterosubtypic virus partially abrogates heterosubtypic immunity in the nose [181]. Cellular responses against conserved viral proteins can provide cross-reactive protection against strains of diverse influenza virus subtypes. The identification of CD4+ T cell antigenic epitopes common to a broad range of influenza A virus strains, including the avian H5N1 virus, will be critical to developing effective heterosubtypic influenza vaccines, particularly since CD4+ T-cell immunity has been clearly established as being required for the generation, maintenance and reactivation of B cell and CTL immune memory.

In this study we found that challenge of mice immunized or infected with influenza virus doesn't change the profile of pre-established immune response, which remains Th2 (characterized by IL-5 and IgG1 antibody production) when mice were immunized with vaccine alone or adjuvanted with MF59, and Th1 (with IFNγ and IgG2a production) in mice previously infected or immunized with vaccine adjuvanted with MF59 + CpG. Although it is known that viral infection promotes a Th1-type immune response, our data demonstrated that it doesn't interfere with the quality of the immune response established by previous vaccination. This is an interesting finding, which allowed us to hypothesize that a pre-existing immunity against the virus could influence the outcome of vaccination. All the results shown in this work were obtained by using immunologically naïve mice, a situation that mimics the condition of infants, which usually have never been in contact with the influenza viruses. Young children appear to be a group for which the use of adjuvants could offer significant advantages, as they often respond suboptimally to Flu vaccines, mainly due to an immature immune system. Perhaps the standard mouse model, involving young animals with no pre-exposure to influenza virus, will prove to be an accurate predictor of adjuvant responses in young children. Nevertheless, the safety of adjuvants needs to be carefully evaluated in this population, although data on the safety of MF59 in toddlers, infants, and newborns are very encouraging [32]. In addition, naïve mice could also be an accurate predictor of human response to pandemic Flu vaccines, as there is no pre-exposure to the relevant strains; however, more studies are needed to confirm this. Given the preferential induction of Th2-biased responses by conventional vaccines in infants and the finding that pre-existing immunity influences the outcome of viral infection, vaccination of infants against seasonal influenza might prevent the induction of immune responses that would otherwise be induced by natural infection (with

type-1 profile) and that are known to be effective against reinfection. In this regard, the use of adjuvants, and in particular the co-delivery of MF59 with an immune-potentiator, is thought to increase vaccine effectiveness in young children by promoting potent Th1 cellular and humoral responses.

However, the immune status in adult humans before the vaccination is very different: humans are annually exposed to the circulating strains of influenza virus, thus a Flu-specific immunity is already present at the time of vaccination. Additional investigations, in which the immune response to vaccine formulations in mice previously exposed to Flu virus (a condition which mimics human experience) was compared to immune responses induced in naïve mice, confirmed that pre-exposure to virus influences the outcome of immune response to vaccine, by biasing it toward a Th1 profile which is maintained also after immunization with MF59 adjuvanted vaccine. Our data support and explain the recent observation that MF59 adjuvanted vaccine promotes the generation of CD4+ T lymphocytes with a Th1-prone effector/memory phenotype in humans [227], a finding that up to now was in contrast with most studies in Balb/c mice where MF59 has been reported to induce a Th2 response. The Th1 profile of immune response induced by vaccination with MF59 adjuvanted vaccine could depend on the pre-existing immunity to Flu virus which is type-1 biased. Our results highlight the need to improve the predictability of the mouse infection model, by using mice previously exposed to virus or to other, unrelated pathogens. Interestingly, the ability of MF59 adjuvant to enhance cellular and humoral immune responses to Flu vaccine is confirmed also in pre-exposed mice.

Development of safe and effective vaccines composed of subunit antigens will require the ability to selectively drive appropriate protective immune responses to them. The use of immunologic adjuvants to enhance and direct immune responses to subunit vaccines is a critical component of a rational vaccine design. Adjuvants have diverse mechanisms of action and must be selected for use on the basis of the immune responses that contribute to the induction of protective immunity. Adjuvants can improve the performance of vaccines by eliciting cytokines that direct Th1 or Th2 immune responses, promoting cell-mediated and humoral immunity, and reducing the number of immunizations or the amount of antigen required for protective immunization.

As adjuvant for seasonal and potential pandemic vaccines, MF59 could allow a significant reduction in the antigen dose, while maintaining the potency of the vaccine, a finding that

might be important to allow an increase in the number of people immunized when an influenza pandemic occurs, assuming vaccine is available [162].

The use of MF59 adjuvant and its combinations with immune-potentiators possibly allow a further reduction of the antigen dose required to stimulate the appropriate immune response able to create effective immunity against specific diseases [148, 162, 228 and 229]. Accumulated data clearly establish that MF59 is a more potent adjuvant than Alum for a range of vaccines, while having a similarly acceptable safety profile in humans [169, 189 and 229]. Also in the context of pediatric vaccines, MF59 was shown to be a well-tolerated and potent adjuvant [189, 190 and 230].

The use of a combination of adjuvants with additive and sometimes synergistic effects, provides a potential advantage over the conventional use of a single adjuvant. The immune potentiator CpG, like other TLR agonists, presents an attractive tool for disease targets such as influenza and other infections with virus or intracellular pathogens that require enhanced Th1 immune responses, including the induction of IFNγ and TNFα. Importantly, the co-delivery of some TLR agonists (i.e. E6020 or IC31) with MF59 emulsion allows a finer tuning towards a particular Th bias likely improving the overall efficacy of the vaccine. It remains to be evaluated if the respective combinations retain their remarkable potency while also being safe and well tolerated in humans.

6. BIBLIOGRAPHY

[1] **Martin PM, Martin-Granel E.** 2,500-year evolution of the term epidemic.
Emerg Infect Dis. 2006 Jun; 12(6):976-80.

[2] **Hippocrates (translated by Francis Adams).** Of the Epidemics.
Written 400 B.C.E. Retrieved 2006-10-18.

[3] **Doherty PC, Turner SJ, Webby RG, Thomas PG.** Influenza and the challenge for immunology.
Nat Immunol. 2006 May; 7(5):449-55.

[4] **Wright, P.F. & Webster, R.G.** In Fields Virology, Orthomyxoviruses, 4th edn. (eds. Knipe, D.M. & Howley, P.M.) 1533–1579 (Lippincott Williams & Wilkins, Philadelphia, 2001).

[5] **Holmes EC, Ghedin E, Miller N, Taylor J, Bao Y, St George K, Grenfell BT, Salzberg SL, Fraser CM, Lipman DJ, Taubenberger JK.** Whole-genome analysis of human influenza A virus reveals multiple persistent lineages and reassortment among recent H3N2 viruses.
PLoS Biol. 2005 Sep; 3(9):e300.

[6] **Nicholson KG, Wood JM, Zambon M.** Influenza.
The Lancet. 2003; 362:1733-45.

[7] **Turner D, Wailoo A, Nicholson K, Cooper N, Sutton A, Abrams K.** Systematic review and economic decision modelling for the prevention and treatment of influenza A and B.
Health Technol Assess. 2003;7(35):iii-iv, xi-xiii, 1-170.

[8] **Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, Fukuda K.** Mortality associated with influenza and respiratory syncytial virus in the United States.
JAMA. 2003 Jan 8; 289(2):179-86.

[9] **Stephenson, I; Nicholson, KG.** Influenza: vaccination and treatment.
Eur.Respir.J. 2001; 17:1282-1293.

[10] **Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, Smith C, Hill RC, Ferro P, Pompey J, Bright RA, Medina MJ, Johnson CM, Olsen CW, Cox NJ, Klimov AI, Katz JM, Donis RO.** Transmission of equine influenza virus to dogs.
Science. 2005 Oct 21; 310(5747):482-5.

[11] **Gyarmati P, Conze T, Zohari S, LeBlanc N, Nilsson M, Landegren U, Banér J, Belák S.** Simultaneous genotyping of all haemagglutinin and neuraminidase subtypes of avian influenza viruses by use of padlock probes.
J Clin Microbiol. 2008 May;46(5):1747-51.

[12] **Lamb RA and Krug RM.** Orthomyxoviridae: the viruses and their replication.
In Fields Virology 4th edn. (eds. Knipe, D.M. & Howley, P.M.) 1533–1579 (Lippincott Williams & Wilkins, Philadelphia, 2001).

[13] **Wagner R, Matrosovich M, Klenk H.** Functional balance between haemagglutinin and neuraminidase in influenza virus infections.
Rev Med Virol. 2002 May-Jun; 12(3):159-66.

[14] **Lakadamyali M, Rust MJ, Babcock HP, Zhuang X.** Visualizing infection of individual influenza viruses.
Proc Natl Acad Sci U S A. 2003 Aug 5; 100(16):9280-5.

[15] **Nayak D, Hui E, Barman S.** Assembly and budding of influenza virus.
Virus Res. 2004 Dec; 106(2):147-65.

[16] **Finkenstädt BF, Morton A, Rand DA.** Modelling antigenic drift in weekly flu incidence.
Stat Med. 2005 Nov 30; 24(22):3447-61.

[17] **Koelle K, Cobey S, Grenfell B, Pascual M.** Epochal Evolution Shapes the Phylodynamics of Interpandemic Influenza A (H3N2) in Humans.
Science. 2006 Dec 22; 314(5807):1898-903.

[18] **Fitch WM, Leiter JM, Li XQ, Palese P.** Positive Darwinian evolution in human influenza A viruses.
Proc Natl Acad Sci U S A. 1991 May 15; 88(10):4270-4.

[19] **Boni MF, Gog JR, Andreasen V, Christiansen FB.** Influenza drift and epidemic size: the race between generating and escaping immunity.
Theor Popul Biol. 2004 Mar; 65(2):179-91.

[20] **Cox RJ, Brokstad KA, Ogra P.** Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines.
Scand J Immunol. 2004 Jan; 59(1):1-15.

[21] **Webby RJ, Webster RG.** Emergence of influenza A viruses.
Philos Trans R Soc Lond B Biol Sci. 2001 Dec 29; 356(1416):1817-28.

[22] **Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y.** Evolution and ecology of influenza A viruses.
Microbiol Rev. 1992 Mar; 56(1):152-79.

[23] **Akira S, Uematsu S, Takeuchi O.** Pathogen recognition and innate immunity.
Cell. 2006 Feb 24; 124(4):783-801.

[24] **Christensen JE, Thomsen AR.** Co-ordinating innate and adaptive immunity to viral infection: mobility is the key.
APMIS. 2009 May; 117(5-6):338-55.

[25] **Banchereau J, Steinman RM.** Dendritic cells and the control of immunity.
Nature. 1998 Mar 19; 392(6673):245-52.

[26] **Ridge JP, Di Rosa F, Matzinger P.** A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell.
Nature. 1998 Jun 4; 393(6684):474-8.

[27] **Lee HK, Iwasaki A.** Innate control of adaptive immunity: dendritic cells and beyond.
Semin Immunol. 2007 Feb; 19(1):48-55.

[28] **White MR, Doss M, Boland P, Tecle T, Hartshorn KL.** Innate immunity to influenza virus: implications for future therapy.
Expert Rev Clin Immunol. 2008 July 1; 4(4): 497–514.

[29] **Le Goffic R, Pothlichet J, Vitour D, Fujita T, Meurs E, Chignard M, Si-Tahar M.** Cutting Edge: Influenza A virus activates TLR3-dependent inflammatory and RIG-I-dependent antiviral responses in human lung epithelial cells.
J Immunol. 2007 Mar 15; 178(6):3368-72.

[30] **Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, Si-Tahar M.** Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus.
J Biol Chem. 2005 Feb 18; 280(7):5571-80.

[31] **Zeng H, Goldsmith C, Thawatsupha P, Chittaganpitch M, Waicharoen S, Zaki S, Tumpey TM and Katz JM.** Highly Pathogenic Avian Influenza H5N1 Viruses Elicit an Attenuated Type I Interferon Response in Polarized Human Bronchial Epithelial Cells.
J Virol. 2007 Nov; 81(22): 12439–12449.

[32] **Chan MC, Cheung CY, Chui WH, Tsao SW, Nicholls JM, Chan YO, Chan RW, Long HT, Poon LL, Guan Y, Peiris JS.** Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells.
Respir Res. 2005 Nov 11; 6:135.

[33] **Hashimoto Y, Moki T, Takizawa T, Shiratsuchi A, Nakanishi Y.** Evidence for phagocytosis of influenza virus-infected, apoptotic cells by neutrophils and macrophages in mice.
J Immunol. 2007 Feb 15; 178(4):2448-57.

[34] **Arndt U, Wennemuth G, Barth P, Nain M, Al-Abed Y, Meinhardt A, Gemsa D and Bacher M.** Release of Macrophage Migration Inhibitory Factor and CXCL8/Interleukin-8 from Lung Epithelial Cells Rendered Necrotic by Influenza A Virus Infection.
J Virol. 2002 September; 76(18): 9298–9306.

[35] **Meyerholz DK, Kawashima K, Gallup JM, Grubor B, Ackermann MR.** Expression of select immune genes (surfactant proteins A and D, sheep beta defensin 1, and toll-like receptor 4) by respiratory epithelia is developmentally regulated in the preterm neonatal lamb.
Dev Comp Immunol. 2006; 30(11):1060-9.

[36] **Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S.** Host innate immune receptors and beyond: making sense of microbial infections.
Cell Host Microbe. 2008 Jun 12; 3(6):352-63.

[37] **Diebold SS, Kaisho T, Hemmi H, Akira S, Reis and Sousa C.** Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA.
Science. 2004 Mar 5; 303(5663):1529-31.

[38] **Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA.** Recognition of single-stranded RNA viruses by Toll-like receptor 7.
Proc Natl Acad Sci U S A. 2004 Apr 13; 101(15):5598-603.

[39] **Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S.** Cell type-specific involvement of RIG-I in antiviral response.
Immunity. 2005 Jul; 23(1):19-28.

[40] **Sinai AP, Webster P, Joiner KA.** Association of host cell endoplasmic reticulum and mitochondria with the Toxoplasma gondii parasitophorous vacuole membrane: a high affinity interaction.
J Cell Sci. 1997 Sep; 110 (Pt 17):2117-28.

[41] **Julkunen I, Melén K, Nyqvist M, Pirhonen J, Sareneva T, Matikainen S.** Inflammatory responses in influenza A virus infection.
Vaccine. 2000 Dec 8; 19 Suppl 1:S32-7.

[42] **Chaudhuri N, Sabroe I.** Basic science of the innate immune system and the lung.
Paediatr Respir Rev. 2008 Dec; 9(4):236-42.

[43] **Watanabe Y, Hashimoto Y, Shiratsuchi A, Takizawa T, Nakanishi Y.** Augmentation of fatality of influenza in mice by inhibition of phagocytosis.
Biochem Biophys Res Commun. 2005 Nov 25; 337(3):881-6.

[44] **Cooper MA, Fehniger TA, Caligiuri MA.** The biology of human natural killer-cell subsets.
Trends Immunol. 2001 Nov; 22(11):633-40.

[45] **Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, Biassoni R, Moretta L.** Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis.
Annu Rev Immunol. 2001; 19:197-223.

[46] **Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Suter M, Perricaudet M, Tursz T, Maraskovsky E, Zitvogel L.** Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo.
Nat Med. 1999 Apr; 5(4):405-11.

[47] **Lucas M, Schachterle W, Oberle K, Aichele P, Diefenbach A.** Dendritic cells prime natural killer cells by trans-presenting interleukin 15.
Immunity. 2007 Apr; 26(4):503-17.

[48] **Ferlazzo G, Pack M, Thomas D, Paludan C, Schmid D, Strowig T, Bougras G, Muller WA, Moretta L and Münz C.** Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs.
Proc Natl Acad Sci U S A. 2004 Nov 23; 101(47): 16606–16611.

[49] **Orange JS, Biron CA.** An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections.
J Immunol. 1996 Feb 1; 156(3):1138-42.

[50] **Orange JS, Biron CA.** Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection.
J Immunol. 1996 Jun 15; 156(12):4746-56.

[51] **Piccioli D, Sbrana S, Meandri E and Valiante NM.** Contact-dependent Stimulation and Inhibition of Dendritic Cells by Natural Killer Cells.
J Exp Med. 2002 February 4; 195(3): 335–341.

[52] **Gerosa F, Baldani-Guerra B, Nisii C, Marchesini V, Carra G, Trinchieri G.** Reciprocal activating interaction between natural killer cells and dendritic cells.
J Exp Med. 2002 Feb 4; 195(3):327-33.

[53] **Eagle RA, Trowsdale J.** Promiscuity and the single receptor: NKG2D.
Nat Rev Immunol. 2007 Sep; 7(9):737-44

[54] **Raulet DH.** Roles of the NKG2D immunoreceptor and its ligands.
Nat Rev Immunol. 2003 Oct; 3(10):781-90.

[55] **Achdout H, Manaster I, Mandelboim O.** Influenza virus infection augments NK cell inhibition through reorganization of major histocompatibility complex class I proteins.
J Virol. 2008 Aug; 82(16):8030-7.

[56] **Banchereau J, Steinman RM.** Dendritic cells and the control of immunity.
Nature. 1998 Mar 19; 392(6673):245-52.

[57] **Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K.** Immunobiology of dendritic cells.
Annu Rev Immunol. 2000; 18:767-811.

[58] **Banchereau J, Steinman RM.** Dendritic cells and the control of immunity.
Nature. 1998 Mar 19; 392(6673):245-52.

[59] **O'Doherty U, Peng M, Gezelter S, Swiggard WJ, Betjes M, Bhardwaj N, Steinman RM.** Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature.
Immunology. 1994 Jul; 82(3):487-93.

[60] **Fernandez-Sesma A, Marukian S, Ebersole BJ, Kaminski D, Park MS, Yuen T, Sealfon SC, García-Sastre A, Moran TM.** Influenza virus evades innate and adaptive immunity via the NS1 protein.
J Virol. 2006 Jul; 80(13):6295-304.

[61] **Kohlmeier JE and Woodland DL.** Immunity to Respiratory Viruses.
Annu. Rev. Immunol. 2009. 27: 61-82.

[62] **Abbas AK, Murphy KM, Sher A.** Functional diversity of helper T lymphocytes.
Nature. 1996 Oct 31; 383(6603):787-93.

[63] **Cella M, Facchetti F, Lanzavecchia A, Colonna M.** Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization.
Nat Immunol. 2000 Oct; 1(4):305-10.

[64] **Gerhard W, Mozdzanowska K, Furchner M, Washko G, Maiese K.** Role of the B-cell response in recovery of mice from primary influenza virus infection.
Immunol Rev. 1997 Oct; 159:95-103.

[65] **Brown DM, Dilzer AM, Meents DL and Swain SL.** CD4 T Cell-Mediated Protection from Lethal Influenza: Perforin and Antibody-Mediated Mechanisms Give a One-Two Punch1.
The Journal of Immunology, 2006, 177: 2888-2898.

[66] **Flynn KJ, Belz GT, Altman JD, Ahmed R, Woodland DL, Doherty PC.** Virus-specific $CD8^+$ T cells in primary and secondary influenza pneumonia.
Immunity. 1998. 8:683–91.

[67] **Hou S, Doherty PC.** Clearance of Sendai virus by CD8+ T cells requires direct targeting to virus-infected epithelium.
Eur. J. Immunol. 1995. 25:111–16

[68] **Topham DJ, Tripp RA, Doherty PC.** CD8+ T cells clear influenza virus by perforin or Fas-dependent processes.
J Immunol. 1997 Dec 1; 159(11):5197-200.

[69] **Graham MB, Braciale TJ.** Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice.
J Exp Med. 1997 Dec 15; 186(12):2063-8.

[70] **Lee BO, Rangel-Moreno J, Moyron-Quiroz JE, Hartson L, Makris M, Sprague F, Lund FE, Randall TD.** CD4 T cell-independent antibody response promotes resolution of primary influenza infection and helps to prevent reinfection.
J Immunol. 2005 Nov 1; 175(9):5827-38.

[71] **McHeyzer-Williams LJ, Pelletier N, Mark L, Fazilleau N, McHeyzer-Williams MG.** Follicular helper T cells as cognate regulators of B cell immunity.
Curr Opin Immunol. 2009 Jun; 21(3):266-73.

[72] **Ghetie V, Hubbard JG, Kim JK, Tsen MF, Lee Y, Ward ES.** Abnormally short serum half-lives of IgG in beta 2-microglobulin-deficient mice.
Eur J Immunol. 1996 Mar; 26(3):690-6.

[73] **Hou S, Doherty PC, Zijlstra M, Jaenisch R, Katz JM.** Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells.
J Immunol. 1992 Aug 15; 149(4):1319-25.

[74] **Law M and Hangartner L.** Antibodies against viruses: passive and active immunization.
Curr Opin Immunol. 2008 August; 20(4): 486–492.

[75] **Janeway CA, P Travers, M Walport, MJ Shlomchik.** Immunobiology 5. 2001. New York: Garland Publishing.

[76] **Sasaki S, He XS, Holmes TH, Dekker CL, Kemble GW, Arvin AM, Greenberg HB.** Influence of prior influenza vaccination on antibody and B-cell responses.
PLoS One. 2008 Aug 20; 3(8):e2975.

[77] **Sasaki S, Jaimes MC, Holmes TH, Dekker CL, Mahmood K, Kemble GW, Arvin AM, Greenberg HB.** Comparison of the influenza virus-specific effector and memory B-cell responses to immunization of children and adults with live attenuated or inactivated influenza virus vaccines.
J Virol. 2007 Jan; 81(1):215-28. Epub 2006 Oct 18.

[78] **Zeman AM, Holmes TH, Stamatis S, Tu W, He XS, Bouvier N, Kemble G, Greenberg HB, Lewis DB, Arvin AM, Dekker CL.** Humoral and cellular immune responses in children given annual immunization with trivalent inactivated influenza vaccine.
Pediatr Infect Dis J. 2007 Feb; 26(2):107-15.

[79] **Plotkin JB, Dushoff J, Levin SA.** Hemagglutinin sequence clusters and the antigenic evolution of influenza A virus.
Proc Natl Acad Sci U S A. 2002; 99:6263–6268.

[80] **McDonald NJ, Smith CB, Cox NJ.** Antigenic drift in the evolution of H1N1 influenza A viruses resulting from deletion of a single amino acid in the haemagglutinin gene.
J Gen Virol. 2007 Dec; 88(Pt 12):3209-13.

[81] **Krystal M, Young JF, Palese P, Wilson IA, Skehel JJ, et al.** Sequential Mutations in Hemagglutinins of Influenza B Virus Isolates: Definition of Antigenic Domains.
Proc Natl Acad Sci U S A. 1983; 80:4527–4531.

[82] **Subbarao K, Murphy BR, Fauci AS.** Development of effective vaccines against pandemic influenza.
Immunity. 2006 Jan; 24(1):5-9.

[83] **Rimmelzwaan GF, Osterhaust AD.** Influenza vaccines: new developments.
Curr Opin Pharmacol. 2001 Oct; 1(5):491-6.

[84] **Nolan T, Lee MS, Cordova JM, Cho I, Walker RE, August MJ, Larson S, Coelingh KL, Mendelman PM.** Safety and immunogenicity of a live-attenuated influenza vaccine blended and filled at two manufacturing facilities.
Vaccine. 2003 Mar 7; 21(11-12):1224-31.

[85] **Nichol KL.** Efficacy/clinical effectiveness of inactivated influenza virus vaccines in adults.
In Textbook of influenza. London: Blackwell Science; 1998. pp. 358–372. (eds. Nicholson KG, Webster RG, Hay AJ).

[86] **Palese P.** Making better influenza virus vaccines?
Emerg Infect Dis. 2006 Jan; 12(1):61-5.

[87] **Neumann G, Kawaoka Y.** Reverse genetics of influenza virus.
Virology. 2001 Sep 1; 287(2):243-50.

[88] **Osterhaus A, Thais DS, Otavio O.** Preparing for the Influenza Pandemic.
In Recent Advances in Immunization, 2nd Edition, pp. 99-113(15)

[89] **Sambhara S., Stephenson I.** Moving influenza vaccines forward.
Expert Rev. Vaccines 8 (4), 375-377 (2009)

[90] **Ulmer JB, Valley U, Rappuoli R.** Vaccine manufacturing: challenges and solutions.
Nat Biotechnol. 2006 Nov; 24(11):1377-83.

[91] **Palache AM, Scheepers HS, de Regt V, van Ewijk P, Baljet M, Brands R, van Scharrenburg GJ.** Safety, reactogenicity and immunogenicity of Madin Darby Canine Kidney cell-derived inactivated influenza subunit vaccine. A meta-analysis of clinical studies.
Dev Biol Stand. 1999; 98:115-25; discussion 133-4.

[92] **Merten OW, Hannoun C, Manuguerra JC, Ventre F, Petres S.** Production of influenza virus in cell cultures for vaccine preparation.
Adv Exp Med Biol. 1996; 397:141-51.

[93] **Kessler N, Thomas-Roche G, Gérentes L, Aymard M.** Suitability of MDCK cells grown in a serum-free medium for influenza virus production.
Dev Biol Stand. 1999; 98:13-21; discussion 73-4.

[94] **Merten OW, Manuguerra JC, Hannoun C, van der Werf S.** Production of influenza virus in serum-free mammalian cell cultures.
Dev Biol Stand. 1999; 98:23-37; discussion 73-4.

[95] **Murakami S, Horimoto T, Mai le Q, Nidom CA, Chen H, Muramoto Y, Yamada S, Iwasa A, Iwatsuki-Horimoto K, Shimojima M, Iwata A, Kawaoka Y.** Growth determinants for H5N1 influenza vaccine seed viruses in MDCK cells.
J Virol. 2008 Nov; 82(21):10502-9. Epub 2008 Sep 3.

[96] **Govorkova EA, Kodihalli S, Alymova IV, Fanget B, Webster RG.** Growth and immunogenicity of influenza viruses cultivated in Vero or MDCK cells and in embryonated chicken eggs.
Dev Biol Stand. 1999; 98:39-51; discussion 73-4.

[97] **Robertson JS, Cook P, Attwell AM, Williams SP.** Replicative advantage in tissue culture of egg-adapted influenza virus over tissue-culture derived virus: implications for vaccine manufacture.
Vaccine. 1995 Nov; 13(16):1583-8.

[98] **Harari A, Dutoit V, Cellerai C, Bart PA, Du Pasqiuer RA, Pantaleo G.** Functional signatures of protective antiviral T-cell immunity in human virus infections.
Immunol.Rev. 2006; 211:236-251.

[99] **Chen D, Periwal SB, Larrivee K, Zuleger C, Erickson CA, Endres RL and Payne LG.** Serum and Mucosal Immune Responses to an Inactivated Influenza Virus Vaccine Induced by Epidermal Powder Immunization.
J Virol. 2001 September; 75(17): 7956–7965.

[100] **Epstein SL, Kong WP, Misplon JA, Lo CY, Tumpey TM, Xu L, Nabel GJ.** Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein.
Vaccine. 2005 Nov 16; 23(46-47):5404-10. Epub 2005 Jun 13.

[101] **Thomas PG, Keating R, Hulse-Post DJ, Doherty PC.** Cell-mediated protection in influenza infection.
Emerg Infect Dis. 2006 Jan; 12(1):48-54.

[102] **Townsend AR, Skehel JJ.** The influenza A virus nucleoprotein gene controls the induction of both subtype specific and cross-reactive cytotoxic T cells.
J Exp Med. 1984 Aug 1; 160(2):552-63.

[103] **Yewdell JW, Bennink JR, Smith GL, Moss B.** Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes.
Proc Natl Acad Sci U S A. 1985 Mar; 82(6):1785-9.

[104] **Townsend AR, Rothbard J, Gotch FM, Bahadur G, Wraith D, McMichael AJ.**The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides.
Cell. 1986 Mar 28; 44(6):959-68.

[105] **Gotch F, McMichael A, Smith G, Moss B.** Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes.
J Exp Med. 1987 Feb 1; 165(2):408-16.

[106] **Thomas PG, Keating R, Hulse-Post DJ, Doherty PC.** Cell-mediated Protection in Influenza Infection.
Emerg. Infect. Dis. 2006; 12 (1): 48-54.

[107] **Epstein SL, Tumpey TM, Misplon JA, Lo CY, Cooper LA, Subbarao K, Renshaw M, Sambhara S, Katz JM.** DNA vaccine expressing conserved influenza virus proteins protective against H5N1 challenge infection in mice.
Emerg Infect Dis. 2002 Aug; 8(8):796-801.

[108] **Doherty PC, Kelso A.** Toward a broadly protective influenza vaccine.
J Clin Invest. 2008 Oct; 118(10):3273-5.

[109] **Keitel WA, Atmar RL, Cate TR, Petersen NJ, Greenberg SB, Ruben F, Couch RB.** Safety of high doses of influenza vaccine and effect on antibody responses in elderly persons.
Arch Intern Med. 2006 May 22; 166(10):1121-7.

[110] **Auewarakul P, Kositanont U, Sornsathapornkul P, Tothong P, Kanyok R, Thongcharoen P.** Antibody responses after dose-sparing intradermal influenza vaccination.
Vaccine. 2007 Jan 8;25(4):659-63.

[111] **Ansaldi F, Canepa P, Parodi V, Bacilieri S, Orsi A, Compagnino F, Icardi G, Durando P.** Adjuvanted seasonal influenza vaccines and perpetual viral metamorphosis: the importance of cross-protection.
Vaccine. 2009 May 26; 27(25-26):3345-8. Epub 2009 Feb 5.

[112] **Pashine A, Valiante NM, Ulmer JB.** Targeting the innate immune response with improved vaccine adjuvants.
Nat Med. 2005 Apr; 11(4 Suppl):S63-8.

[113] **Hoebe K, Janssen E, Beutler B.** The interface between innate and adaptive immunity.
Nat Immunol. 2004 Oct; 5(10):971-4.

[114] **O'Hagan DT.** Recent developments in vaccine delivery systems.
Curr Drug Targets Infect Disord. 2001 Nov; 1(3):273-86.

[115] **Guy B.** The perfect mix: recent progress in adjuvant research.
Nat Rev Microbiol. 2007 Jul; 5(7):505-17.

[116] **Fraser CK, Diener KR, Brown MP, Hayball JD.** Improving vaccines by incorporating immunological coadjuvants.
Expert Rev Vaccines. 2007 Aug; 6(4):559-78.

[117] **Tritto E, Mosca F, De Gregorio E.** Mechanism of action of licensed vaccine adjuvants.
Vaccine. 2009 May 26; 27(25-26):3331-4.

[118] **Boland G, Beran J, Lievens M, Sasadeusz J, Dentico P, Nothdurft H, Zuckerman JN, Genton B, Steffen R, Loutan L, Van Hattum J, Stoffel M.** Safety and immunogenicity profile of an experimental hepatitis B vaccine adjuvanted with AS04.
Vaccine. 2004 Dec 2; 23(3):316-20.

[119] **Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM.** CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma.
Proc Natl Acad Sci U S A. 1996 Apr 2; 93(7):2879-83.

[120] **Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S.** A Toll-like receptor recognizes bacterial DNA.
Nature. 2000 Dec 7; 408(6813):740-5.

[121] **Klinman DM, Barnhart KM, Conover J.** CpG motifs as immune adjuvants.
Vaccine. 1999 Jan; 17(1):19-25.

[122] **Krieg AM, Efler SM, Wittpoth M, Al Adhami MJ, Davis HL.** Induction of systemic TH1-like innate immunity in normal volunteers following subcutaneous but not intravenous administration of CPG 7909, a synthetic B-class CpG oligodeoxynucleotide TLR9 agonist.
J Immunother. 2004 Nov-Dec; 27(6):460-71.

[123] **Krieg AM.** CpG motifs in bacterial DNA and their immune effects.
Annu Rev Immunol. 2002; 20:709-60.

[124] **Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM.** CpG motifs in bacterial DNA trigger direct B-cell activation.
Nature. 1995. 374(6522):6546–49.

[125] **Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM.** CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen.
J Immunol. 1998 Jan 15; 160(2):870-6.

[126] **Kobayashi H, Horner AA, Takabayashi K, Nguyen MD, Huang E, Cinman N, Raz E.** Immunostimulatory DNA pre-priming: a novel approach for prolonged Th1-biased immunity.
Cell Immunol. 1999 Nov 25; 198(1):69-75.

[127] **Hartmann G, Weiner GJ, Krieg AM.** CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells.
Proc Natl Acad Sci U S A. 1999 Aug 3; 96(16):9305-10.

[128] **Kadowaki N, Antonenko S, Liu YJ.** Distinct CpG DNA and polyinosinic-polycytidylic acid double-stranded RNA, respectively, stimulate CD11c- type 2 dendritic cell precursors and CD11c+ dendritic cells to produce type I IFN.
J Immunol. 2001 Feb 15; 166(4):2291-5.

[129] **Krug A, Towarowski A, Britsch S, Rothenfusser S, Hornung V, Bals R, Giese T, Engelmann H, Endres S, Krieg AM, Hartmann G.** Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12.
Eur J Immunol. 2001 Oct; 31(10):3026-37.

[130] **Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, Liu YJ.** Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens.
J Exp Med. 2001 Sep 17; 194(6):863-9.

[131] **Guiducci C, Ott G, Chan JH, Damon E, Calacsan C, Matray T, Lee KD, Coffman RL and Barrat FJ.** Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation.
J Exp Med. 2006 July. Volume 203, Number 8, 1999-2008

[132] **Hemmi H, Kaisho T, Takeda K, Akira S.** The roles of Toll-like receptor 9, MyD88, and DNA-dependent protein kinase catalytic subunit in the effects of two distinct CpG DNAs on dendritic cell subsets.
J Immunol. 2003 Mar 15; 170(6):3059-64.

[133] **Kerkmann M, Rothenfusser S, Hornung V, Towarowski A, Wagner M, Sarris A, Giese T, Endres S, Hartmann G.** Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells.
J Immunol. 2003 May 1; 170(9):4465-74.

[134] **Krug A, Rothenfusser S, Hornung V, Jahrsdörfer B, Blackwell S, Ballas ZK, Endres S, Krieg AM, Hartmann G.** Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells.
Eur J Immunol. 2001 Jul; 31(7):2154-63.

[135] **Verthelyi D, Kenney RT, Seder RA, Gam AA, Friedag B, Klinman DM.** CpG oligodeoxynucleotides as vaccine adjuvants in primates.
J Immunol. 2002 Feb 15; 168(4):1659-63.

[136] **Hartmann G, Battiany J, Poeck H, Wagner M, Kerkmann M, Lubenow N, Rothenfusser S, Endres S.** Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells
Eur J Immunol. 2003 Jun; 33(6):1633-41.

[137] **Wack A, Baudner BC, Hilbert AK, Manini I, Nuti S, Tavarini S, Scheffczik H, Ugozzoli M, Singh M, Kazzaz J, Montomoli E, Del Giudice G, Rappuoli R, O'Hagan DT.** Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice
Vaccine. 2008 Jan 24; 26(4):552-61.

[138] **Singh M, Ott G, Kazzaz J, Ugozzoli M, Briones M, Donnelly J, O'Hagan DT.** Cationic microparticles are an effective delivery system for immune stimulatory CpG DNA.
Pharm Res. 2001 Oct; 18(10):1476-9.

[139] **Schellack C, Prinz K, Egyed A, Fritz JH, Wittmann B, Ginzler M, Swatosch G, Zauner W, Kast C, Akira S, von Gabain A, Buschle M, Lingnau K.** IC31, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses.
Vaccine. 2006 Jun 29; 24(26):5461-72.

[140] **J.H. Fritz, S. Brunner, M.L. Birnstiel, M. Buschle, A. Gabain and F. Mattner et al.** The artificial antimicrobial peptide KLKLLLLLKLK induces predominantly a TH2-type immune response to co-injected antigens.
Vaccine 22 (25–26) (2004), pp. 3274–3284.

[141] **Riedl K, Riedl R, von Gabain A, Nagy E, Lingnau K.** The novel adjuvant IC31 strongly improves influenza vaccine-specific cellular and humoral immune responses in young adult and aged mice.
Vaccine. 2008 Jun 25; 26(27-28):3461-8.

[142] **Kenney RT, Edelman R.** Survey of human-use adjuvants.
Expert Rev Vaccines. 2003 Apr; 2(2):167-88.

[143] **Lipford GB, Sparwasser T, Bauer M, Zimmermann S, Koch ES, Heeg K, Wagner H.** Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines.
Eur J Immunol. 1997 Dec; 27(12):3420-6.

[144] **Zinkernagel RM.** Localization dose and time of antigens determine immune reactivity.
Semin Immunol. 2000 Jun; 12(3):163-71; discussion 257-344.

[145] **Evans JT, Cluff CW, Johnson DA, Lacy MJ, Persing DH, Baldridge JR.** Enhancement of antigen-specific immunity via the TLR4 ligands MPL adjuvant and Ribi.529.
Expert Rev Vaccines. 2003 Apr; 2(2):219-29.

[146] CERVARIX® Product Information Human Papillomavirus Vaccine Types 16 and 18 (Recombinant, AS04 adjuvanted) GlaxoSmithKline Australia Pty Ltd. (2007).

[147] **Kundi M.** New hepatitis B vaccine formulated with an improved adjuvant system.
Expert Rev Vaccines. 2007 Apr; 6(2):133-40.

[148] **Garçon N, Chomez P, Van Mechelen M.** GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives.
Expert Rev Vaccines. 2007 Oct; 6(5):723-39.

[149] **Przetak M, Chow J, Cheng H, Rose J, Hawkins LD, Ishizaka ST.** Novel synthetic LPS receptor agonists boost systemic and mucosal antibody responses in mice.
Vaccine. 2003 Feb 14; 21(9-10):961-70.

[150] **Hawkins LD, Ishizaka ST, McGuinness P, Zhang H, Gavin W, DeCosta B, Meng Z, Yang H, Mullarkey M, Young DW, Yang H, Rossignol DP, Nault A, Rose J, Przetak M, Chow JC, Gusovsky F.** A novel class of endotoxin receptor agonists with simplified structure, toll-like receptor 4-dependent immunostimulatory action, and adjuvant activity.
J Pharmacol Exp Ther. 2002 Feb; 300(2):655-61.

[151] **Ishizaka ST, Hawkins LD.** E6020: a synthetic Toll-like receptor 4 agonist as a vaccine adjuvant.
Expert Rev Vaccines. 2007 Oct; 6(5):773-84.

[152] **O'Hagan DT, Valiante NM.** Recent advances in the discovery and delivery of vaccine adjuvants.
Nat Rev Drug Discov. 2003 Sep; 2(9):727-35.

[153] **Gupta RK.** Aluminum compounds as vaccine adjuvants.
Adv Drug Deliv Rev. 1998 Jul 6; 32(3):155-172.

[154] **Hem SL, Hogenesch H.** Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiation.
Expert Rev Vaccines. 2007 Oct; 6(5):685-98.

[155] **Singh M, O'Hagan DT.** Recent advances in vaccine adjuvants.
Pharm Res. 2002 Jun; 19(6):715-28.

[156] **Trinchieri G, Sher A.** Cooperation of Toll-like receptor signals in innate immune defence.
Nat Rev Immunol. 2007 Mar; 7(3):179-90.

[157] **Li H, Nookala S, Re F.** Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release.
J Immunol. 2007 Apr 15; 178(8):5271-6.

[158] **Li H, Willingham SB, Ting JP-Y and Re F.** Cutting Edge: Inflammasome activation by Alum and Alum's adjuvant effect are mediated by NLRP3.
J Immunol. 2008 July 1; 181(1): 17–21.

[159] **O'Hagan D T, Wack A and Podda A.** MF59 Is a Safe and Potent Vaccine Adjuvant for Flu Vaccines in Humans: What Did We Learn During Its Development?
Clin Pharmacol Ther. 2007 Dec; 82(6):740-4.

[160] **Ott G, Barchfeld GL, Chernoff D, Radhakrishnan R, van Hoogevest P, Van Nest G.** MF59. Design and evaluation of a safe and potent adjuvant for human vaccines
Pharm Biotechnol. 1995; 6:277-96.

[161] **Singh M, Ugozzoli M, Kazzaz J, Chesko J, Soenawan E, Mannucci D, Titta F, Contorni M, Volpini G, Del Guidice G, O'Hagan DT.** A preliminary evaluation of alternative adjuvants to alum using a range of established and new generation vaccine antigens.
Vaccine. 2006 Mar 6; 24(10):1680-6.

[162] **Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, Zambon MC.** Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza.
Lancet. 2001 Jun 16; 357(9272):1937-43.

[163] **Stephenson I, Nicholson KG, Colegate A, Podda A, Wood J, Ypma E, Zambon M.** Boosting immunity to influenza H5N1 with MF59-adjuvanted H5N3 A/Duck/Singapore/97 vaccine in a primed human population.
Vaccine. 2003 Apr 2; 21(15):1687-93.

[164] **Dupuis M, Murphy TJ, Higgins D, Ugozzoli M, van Nest G, Ott G, McDonald DM.** Dendritic cells internalize vaccine adjuvant after intramuscular injection.
Cell Immunol. 1998 May 25; 186(1):18-27.

[165] **Dupuis M, Denis-Mize K, LaBarbara A, Peters W, Charo IF, McDonald DM, Ott G.** Immunization with the adjuvant MF59 induces macrophage trafficking and apoptosis.
Eur J Immunol. 2001 Oct; 31(10):2910-8.

[166] **O'Hagan DT.** MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection.
Expert Rev Vaccines. 2007 Oct; 6(5):699-710.

[167] **Baudner BC, Ronconi V, Casini D, Tortoli M, Kazzaz J, Singh M, Hawkins LD, Wack A, O'Hagan DT.** MF59 emulsion is an effective delivery system for a synthetic TLR4 agonist (E6020).
Pharm Res. 2009 Jun; 26(6):1477-85.

[168] **Finney DJ, 1978.** Statistical Method in Biological Assay, third ed. Charles Griffin & Co. Ltd, London, England. 508 pp.

[169] **Ott G, Radhakrishnan R, Fang J-H and Hora M.** The adjuvant MF59: a 10-year perspective.
Methods Mol. Med. 42: 211-228 (2000).

[170] **Dupuis M, McDonald DM, Ott G.** Distribution of adjuvant MF59 and antigen gD2 after intramuscular injection in mice.
Vaccine. 1999 Oct 14; 18(5-6):434-9.

[171] **Holvast A, van Assen S, de Haan A, Huckriede A, Benne CA, Westra J,Palache A, Wilschut J, Kallenberg CGM and Bijl M.** Studies of Cell-Mediated Immune Responses to Influenza Vaccination in Systemic Lupus Erythematosus.
Arthritis Rheum. 2009 Aug; 60(8):2438-47.

[172] **Rimmelzwaana GF, McElhaneyb JE.** Correlates of protection: Novel generations of influenza vaccines.
Vaccine. 2008 Sep 12; 26 Suppl 4:D41-4.

[173] **Palladino G, Scherle PA and Gerhard W.** Activity of CD4+ T-cell clones of type 1 and type 2 in generation of influenza virus-specific cytotoxic responses in vitro.
J Virol. 1991 November; 65(11): 6071-6076.

[174] **De Jong JC, Palache AM, Beyer WE, Rimmelzwaan GF, Boon AC, Osterhaus AD.** Haemagglutination-inhibiting antibody to influenza virus.
Dev Biol (Basel) 2003; 115: 63-73

[175] **Klinman DM, Currie D, Gursel I, Verthelyi D.** Use of CpG oligodeoxynucleotides as immune adjuvants.
Immunol Rev. 2004 Jun; 199:201-16.

[176] **Ishizaka ST, Hawkins LD.** E6020: a synthetic Toll-like receptor 4 agonist as a vaccine adjuvant.
Expert Rev Vaccines. 2007 Oct; 6(5):773-84.

[177] **Olafsdottir TA, Lingnau K, Nagy E, Jonsdottir I.** IC31, a two-component novel adjuvant mixed with a conjugate vaccine enhances protective immunity against pneumococcal disease in neonatal mice.
Scand J Immunol. 2009 Mar; 69(3):194-202.

[178] **Bix M, Wang ZE, Thiel B, Schork NJ and Locksley RM.** Genetic Regulation of Commitment to Interleukin 4 Production by a CD4+ T Cell–intrinsic Mechanism.
J Exp Med. 1998 December 21; 188(12): 2289–2299.

[179] **Johnson PR, Feldman S, Thompson JM, Mahoney JD, Wright PF.** Immunity to influenza A virus infection in young children: a comparison of natural infection, live cold-adapted vaccine, and inactivated vaccine.
J Infect Dis. 1986 Jul; 154(1):121-7.

[180] **Fazekas G, Rosenwirth B, Dukor P, Gergely J, Rajnavölgyi E.** IgG isotype distribution of local and systemic immune responses induced by influenza virus infection.
Eur J Immunol. 1994 Dec; 24(12):3063-7.

[181] **Liang S, Mozdzanowska K, Palladino G, Gerhard W.** Heterosubtypic immunity to influenza type A virus in mice. Effector mechanisms and their longevity.
J Immunol. 1994 Feb 15; 152(4):1653-61.

[182] **Brown DM, Román E, Swain SL.** CD4 T cell responses to influenza infection.
Semin Immunol. 2004 Jun; 16(3):171-7.

[183] **Thomas PG, Keating R, Hulse-Post DJ, Doherty PC.** Cell-mediated protection in influenza infection.
Emerg Infect Dis. 2006 Jan; 12(1):48-54.

[184] **De Gregorio E, Tritto E, Rappuoli R.** Alum adjuvanticity: unraveling a century old mystery.
Eur J Immunol. 2008 Aug; 38(8):2068-71.

[185] **Pichichero ME.** Improving vaccine delivery using novel adjuvant systems.
Hum Vaccin. 2008 Jul-Aug; 4(4):262-70.

[186] **Thönes N, Herreiner A, Schädlich L, Piuko K, Müller M.** A direct comparison of human papillomavirus type 16 L1 particles reveals a lower immunogenicity of capsomeres than viruslike particles with respect to the induced antibody response.
J Virol. 2008 Jun; 82(11):5472-85.

[187] **Lingnau K, Riedl K, von Gabain A.** IC31 and IC30, novel types of vaccine adjuvant based on peptide delivery systems.
Expert Rev Vaccines. 2007 Oct; 6(5):741-6.

[188] **Agger EM, Rosenkrands I, Olsen AW, Hatch G, Williams A, Kritsch C, Lingnau K, von Gabain A, Andersen CS, Korsholm KS, Andersen P.** Protective immunity to tuberculosis with Ag85B-ESAT-6 in a synthetic cationic adjuvant system IC31.
Vaccine. 2006 Jun 29; 24(26):5452-60.

[189] **Podda A, Del Giudice G.** MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile.
Expert Rev Vaccines. 2003 Apr; 2(2):197-203.

[190] **Mitchell DK, Holmes SJ, Burke RL, Duliege AM, Adler SP.** Immunogenicity of a recombinant human cytomegalovirus gB vaccine in seronegative toddlers.
Pediatr Infect Dis J. 2002 Feb; 21(2):133-8.

[191] **Wong GH, Goeddel DV.** Tumour necrosis factors alpha and beta inhibit virus replication and synergize with interferons.
Nature. 1986 Oct 30-Nov 5; 323(6091):819-22.

[192] **Seo SH, Webster RG.** Tumor necrosis factor alpha exerts powerful anti-influenza virus effects in lung epithelial cells.
J Virol. 2002 Feb; 76(3):1071-6.

[193] **Hayden FG, Fritz R, Lobo MC, Alvord W, Strober W and Straus SE.** Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense.
J Clin Invest. 1998 February 1; 101(3): 643–649.

[194] **Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J, Lederman MM, Benito JM, Goepfert PA, Connors M, Roederer M, Koup RA.** HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells.
Blood. 2006 Jun 15; 107(12):4781-9.

[195] **De Rosa SC, Lu FX, Yu J, Perfetto SP, Falloon J, Moser S, Evans TG, Koup R, Miller CJ, Roederer M.** Vaccination in humans generates broad T cell cytokine responses.
J Immunol. 2004 Nov 1; 173(9):5372-80.

[196] **Pantaleo G, Koup RA.** Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know.
Nat Med. 2004 Aug; 10(8):806-10.

[197] **Seubert A, Monaci E, Pizza M, O'Hagan DT, Wack A.** The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells.
J Immunol. 2008 Apr 15; 180(8):5402-12.

[198] **Mosca F, Tritto E, Muzzi A, Monaci E, Bagnoli F, Iavarone C, O'Hagan D, Rappuoli R, De Gregorio E.** Molecular and cellular signatures of human vaccine adjuvants.
Proc Natl Acad Sci U S A. 2008 Jul 29; 105(30):10501-6.

[199] **Huber VC, McKeon RM, Brackin MN, Miller LA, Keating R, Brown SA, Makarova N, Perez DR, Macdonald GH, McCullers JA.** Distinct contributions of vaccine-induced immunoglobulin G1 (IgG1) and IgG2a antibodies to protective immunity against influenza.
Clin Vaccine Immunol. 2006 Sep; 13(9):981-90.

[200] **Ansaldi F, Bacilieri S, Banfi F, Durando P, Sticchi L, Icardi G, Gasparini R.** Neutralizing and hemagglutination-inhibiting activities of antibodies elicited by the 2004-2005 influenza vaccine against drifted viruses.
Clin Vaccine Immunol. 2006 Jan; 13(1):162-4.

[201] **Neuzil KM.** Pandemic influenza vaccine policy--considering the early evidence.
N Engl J Med. 2009 Dec 17; 361(25):e59.

[202] **Farrell HE, Shellam GR.** Protection against murine cytomegalovirus infection by passive transfer of neutralizing and non-neutralizing monoclonal antibodies.
J Gen Virol. 1991 Jan; 72 (Pt 1):149-56.

[203] **Scallan CD, Jiang H, Liu T, Patarroyo-White S, Sommer JM, Zhou S, Couto LB, Pierce GF.** Human immunoglobulin inhibits liver transduction by AAV vectors at low AAV2 neutralizing titers in SCID mice
Blood. 2006 Mar 1; 107(5):1810-7.

[204] **Klasse PJ, Sattentau QJ.** Occupancy and mechanism in antibody-mediated neutralization of animal viruses.
J Gen Virol. 2002 Sep; 83(Pt 9):2091-108.

[205] **McCullough KC, Crowther JR, Butcher RN, Carpenter WC, Brocchi E, Capucci L, De Simone F.** Immune protection against foot-and-mouth disease virus studied using virus-neutralizing and non-neutralizing concentrations of monoclonal antibodies.
Immunology. 1986 Jul; 58(3):421-8.

[206] **Crowe JE Jr, Suara RO, Brock S, Kallewaard N, House F, Weitkamp JH.** Genetic and structural determinants of virus neutralizing antibodies.
Immunol Res. 2001; 23(2-3):135-45.

[207] **Balachandran N, Bacchetti S, and Rawls WE.** Protection against lethal challenge of BALB/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2.
Infect Immun. 1982 September; 37(3): 1132–1137.

[208] **Schmaljohn AL, Johnson ED, Dalrymple JM, Cole GA.** Non-neutralizing monoclonal antibodies can prevent lethal alphavirus encephalitis.
Nature. 1982 May 6; 297(5861):70-2.

[209] **Boere WA, Benaissa-Trouw BJ, Harmsen T, Erich T, Kraaijeveld CA and Snippe H.** Mechanisms of monoclonal antibody-mediated protection against virulent Semliki Forest virus.
J Virol. 1985 May; 54(2): 546–551.

[210] **Lefrancois L.** Protection against lethal viral infection by neutralizing and nonneutralizing monoclonal antibodies: distinct mechanisms of action in vivo.
J Virol. 1984 July; 51(1): 208–214.

[211] **Shore SL, Nahmias AJ, Starr SE, Wood PA and McFarlin DE.** Detection of cell-dependent cytotoxic antibody to cells infected with herpes simplex virus.
Nature. 1974 Sep 27; 251(5473):350–352.

[212] **Kohl S, Cahall DL, Walters DL and Schaffner VE.** Murine Antibody-Dependent Cellular Cytotoxicity to Herpes Simplex Virus-Infected Target Cells.
J Immunol. 1979 Jul; 123(1):25-30.

[213] **Carragher DM, Kaminski DA, Moquin A, Hartson L and Randall TD.** A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus.
J Immunol. 2008 September 15; 181(6): 4168–4176.

[214] **Ambrose CS, Walker RE, Connor EM.** Live attenuated influenza vaccine in children.
Semin Pediatr Infect Dis. 2006 Oct; 17(4):206-12.

[215] **Asahi Y, Yoshikawa T, Watanabe I, Iwasaki T, Hasegawa H, Sato Y, Shimada S, Nanno M, Matsuoka Y, Ohwaki M, Iwakura Y, Suzuki Y, Aizawa C, Sata T, Kurata T, Tamura S.** Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines.
J Immunol. 2002 Mar 15; 168(6):2930-8.

[216] **Asanuma H, Koide F, Suzuki Y, Nagamine T, Aizawa C, Kurata T, Tamura S.** Cross-protection against influenza virus infection in mice vaccinated by combined nasal/subcutaneous administration.
Vaccine. 1995 Jan; 13(1):3-5.

[217] **Chen ZM, Mao JH, Du LZ, Tang YM.** Association of cytokine responses with disease severity in infants with respiratory syncytial virus infection.
Acta Paediatr. 2002; 91(9):914-22.

[218] **Haan L, Verweij WR, Holtrop M, Brands R, van Scharrenburg GJ, Palache AM, Agsteribbe E, Wilschut J.** Nasal or intramuscular immunization of mice with influenza subunit antigen and the B subunit of Escherichia coli heat-labile toxin induces IgA- or IgG-mediated protective mucosal immunity.
Vaccine. 2001 Apr 6; 19(20-22):2898-907.

[219] **Hajishengallis G, Arce S, Gockel CM, Connell TD, Russell MW.** Immunomodulation with enterotoxins for the generation of secretory immunity or tolerance: applications for oral infections.
J Dent Res. 2005 Dec; 84(12):1104-16.

[220] **Harper SA, Fukuda K, Cox NJ, Bridges CB; Advisory Committee on Immunization Practices.** Using live, attenuated influenza vaccine for prevention and control of influenza: supplemental recommendations of the Advisory Committee on Immunization Practices (ACIP).
MMWR Recomm Rep. 2003 Sep 26; 52(RR-13):1-8.

[221] **Basta NE, Halloran ME, Matrajt L, Longini IM Jr.** Estimating influenza vaccine efficacy from challenge and community-based study data.
Am J Epidemiol. 2008 Dec 15; 168(12):1343-52.

[222] **Braciale TJ.** Immunologic recognition of influenza virus-infected cells. II. Expression of influenza A matrix protein on the infected cell surface and its role in recognition by cross-reactive cytotoxic T cells.
J Exp Med. 1977 Sep 1; 146(3):673-89.

[223] **Kees U, Krammer PH.** Most influenza A virus-specific memory cytotoxic T lymphocytes react with antigenic epitopes associated with internal virus determinants.
J Exp Med. 1984 Feb 1; 159(2):365-77.

[224] **Askonas BA, Taylor PM, Esquivel F.** Cytotoxic T cells in influenza infection.
Ann N Y Acad Sci. 1988; 532:230-7.

[225] **McMichael AJ, Gotch F, Cullen P, Askonas B, Webster RG.** The human cytotoxic T cell response to influenza A vaccination.
Clin Exp Immunol. 1981 Feb; 43(2):276-84.

[226] **Scherle PA and Gerhard W.** Functional analysis of influenza-specific helper T cell clones in vivo. T cells specific for internal viral proteins provide cognate help for B cell responses to hemagglutinin.
J Exp Med. 1986 October 1; 164(4): 1114–1128.

[227] **Galli G, Medini D, Borgogni E, Zedda L, Bardelli M, Malzone C, Nuti S, Tavarini S, Sammicheli C, Hilbert AK, Brauer V, Banzhoff A, Rappuoli R, Del Giudice G, Castellino F.** Adjuvanted H5N1 vaccine induces early CD4+ T cell response that predicts long-term persistence of protective antibody levels.
Proc Natl Acad Sci U S A. 2009 Mar 10; 106(10):3877-82.

[228] **Atmar RL, Keitel WA, Patel SM, Katz JM, She D, El Sahly H, Pompey J, Cate TR, Couch RB.** Safety and immunogenicity of nonadjuvanted and MF59-adjuvanted influenza A/H9N2 vaccine preparations.
Clin Infect Dis. 2006 Nov 1; 43(9):1135-42.

[229] **O'Hagan DT, Singh M, Kazzaz J, Ugozzoli M, Briones M, Donnelly J, Ott G.** Synergistic adjuvant activity of immunostimulatory DNA and oil/water emulsions for immunization with HIV p55 gag antigen.
Vaccine. 2002 Sep 10; 20(27-28):3389-98.

[230] **Cunningham CK, Wara DW, Kang M, Fenton T, Hawkins E, McNamara J, Mofenson L, Duliege AM, Francis D, McFarland EJ, Borkowsky W; Pediatric AIDS Clinical Trials Group 230 Collaborators.**
Safety of 2 recombinant human immunodeficiency virus type 1 (HIV-1) envelope vaccines in neonates born to HIV-1-infected women
Clin Infect Dis. 2001 Mar 1; 32(5):801-7.