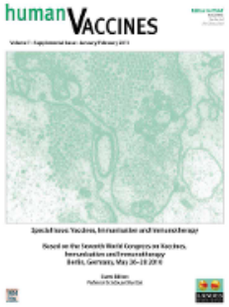

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Adjuvants and alternative routes of administration towards the development of the ideal influenza vaccine

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Key words: influenza vaccines, immunogenicity, cross-reactivity, adjuvants, MF59 oil-in-water emulsion, intradermal administration, microinjection system, universal vaccine

Abbreviations: TIV, trivalent influenza vaccine; HA, hemagglutinin; NA, neuraminidase; WHO, World Health Organization; LAIV, live attenuated influenza vaccine; IM, intramuscular; ID, intradermal; MDCK, Madin-Darby canine kidney; EMA, European Medicines Agency; HIV, human immunodeficiency virus; HI, hemagglutination inhibition; NT, neutralization; CHMP, Committee for Medicinal Products for Human Use; OR, odds ratio; CI, confidence interval; DC, dendritic cell; BCR, B-cell receptor; SRH, single radial hemolysis

Vaccination is universally considered as the principal measure for the control of influenza, which represents a significant burden worldwide, both from a health-care and a socio-economic viewpoint. Conventional non-adjuvanted trivalent influenza vaccines (TIVs) have been recognized as having some deficiencies, such as suboptimal immunogenicity particularly in the elderly, in patients with severe chronic diseases and immunocompromized, indeed, those groups of the population at higher risk of developing severe complications following influenza infection, when compared to healthy adults. Moreover, the protection offered by conventional vaccines may be reduced by periodic antigenic drifts, resulting in a mismatch between the circulating and vaccinal viral strains. Another gap regarding currently available vaccines is related to the egg-based manufacturing system for their production: not only the length of time involved with the latter but also the limited capacity of this platform technology represent a major limitation for the active prevention of influenza, which is particularly important in the case of a new pandemic strain. New technologies used in vaccine composition, administration and manufacture have led to major advances during the last few years and clinical researchers have continued to work hard, investigating several different strategies to improve the performance of influenza vaccines: namely, the addition of different adjuvants (i.e., MF59- and AS03-vaccines, virosomal formulations), the use of alternative routes of administration or manufacture (i.e., intradermal, nasal and oral vaccines and cell culture- and reverse genetic-based vaccines) or of high doses of antigen, and the development of DNA-vaccines, or the use of conserved viral epitopes (i.e., the extracellular portion of the M2 protein, the nucleoprotein and some domains of the hemagglutinin), in the attempt to produce a "universal target" antigen vaccine. The knowledge acquired represents a fundamental challenge for the control of influenza.

An overview of the most recent and interesting results, some of which gained from our own research experience, particularly concerning two successful approaches, of those outlined above, namely the use of: (1) the oil-in-water MF59-adjuvant and (2) the intradermal (ID) route for vaccine administration, through a novel microinjection system, will be reported and discussed, together with the possible implications and perspectives to optimize immunization policies against influenza in the near future.

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The Burden of Influenza and the Rationale for New Approaches in Vaccine Development

Influenza is one of the most important infectious diseases affecting public health in western Countries. Older adults, in particular, are at high risk, as is well documented by the heavy burden of the infection in terms of complications, hospitalizations and deaths occurring during seasonal epidemics.[1,2] Excess admissions are a major problem for health service delivery, and are closely age-related: for example, in England, in a study monitoring hospital admissions during the years 1989–2001, 52% of the 16,227 average annual excess occurred in subjects over 75 years of age, with excess admissions accounting for an average 145,544 bed days annually, two thirds (69%) in the above-mentioned age-target group.[3] Data from the US, collected during the 1990s, confirm this significant impact, with 90% of the 36,000 annual flu-associated respiratory and circulatory-related deaths occurring in individuals aged ≥65 years.[4]

The vulnerability of the elderly to influenza can be explained by the well-known phenomenon of immunosenescence, a para-physiological condition, strictly related to the increase in chronological age, associated with a reduced T-cell activity, affecting

Table 1. Main critical issues and current desiderata for influenza vaccines

Critical issues	Desiderata
Suboptimal immunogenicity and protective efficacy in some target groups:	• Counter age-dependent immune decline
- elderly subjects	• Elicit effective boosting
- patients with underlying chronic disease and immunocompromized	• Create immunological memory
- infants and young children	• Improve priming and carry over
Mismatching between vaccinal and circulating strains (antigenic drift)	• Ensure cross-protection
Annual re-licensure	• Use of conserved viral antigen epitopes
Manufacture in embryonated eggs:	
- duration of the production process (nearly 6 months)	
- difficulties in the growth of the seed strains	• Develope of alternative substrates and/or new technologies for easy, rapid and high vaccine production
- delayed availability in the event of a pandemic	
- limited production capacity dependent upon availability of embryonated eggs	• Allow antigen sparing
- risk of vaccine shortage	
- increasing demand for vaccines worldwide	

both the Th1-humoral and the cytotoxic T-cell function, consequently resulting in a decreased immune response to the antigenic stimulus.[5]

Severe complications following influenza infection have also been reported in patients with chronic underlying diseases (i.e., respiratory, cerebro-cardiovascular, metabolic diseases, immunodeficiencies, etc.,), in subjects presenting specific para-physiological states (i.e., pregnancy), as well as in infants and young children, thus representing other vulnerable categories for which protection is highly desirable.[6,7]

Vaccination is universally considered the principal measure for the control of seasonal influenza, being strongly recommended, in all western Countries, to specific groups within the population at higher risk of infection and complications than healthy adults.[6,7] Currently, most of the prophylactic vaccines against seasonal influenza used worldwide are the subunit and split non-adjuvanted types, the so-called "conventional vaccines," which have been available for more than 60 years,[8] with approximately 300 million doses produced each year.[9] Despite the widespread use of these products, which have significantly contributed to reducing morbidity and mortality rates associated with influenza worldwide, conventional non-adjuvanted trivalent influenza vaccines (TIVs) have also shown some limitations, mainly in terms of immunogenicity in some target-groups of the population, particularly in the elderly and in patients affected with severe diseases or immunocompromized for any reason, for whom immunization is highly recommended, but also in terms of lack of cross-protection against drifted influenza strains.[7,10-18] For this reason, the efficacy of conventional vaccines, in the elderly, is lower than in healthy adults (~70–90%):[7] this finding has led some authors to estimate a clinical protection of these vaccines ranging from 17 to 53% in the elderly depending on the viruses circulating in the community.[19] Based on these observations, some doubts have been raised concerning the real benefit of current vaccination policies in reducing the health-care burden of influenza in this age group.[20]

Another Achilles' heel in the immune prevention of influenza, during the inter-pandemic periods, is represented by the frequent antigenic drift of this virus, which is due to the accumulation of point mutations on the genes encoding the two surface antigenic proteins (hemagglutinin, HA and neuraminidase, NA).[21] Despite annual updates of vaccine composition by the World Health Organization (WHO), the occurrence of this phenomenon, resulting in a mismatch between vaccine and circulating viral strains, can lead to a significant reduction in seroprotection rates in immunized individuals,[17,22,23] with consequently a negative implication in terms of effectiveness of the immunization programs in the community, as reported elsewhere.[24–26]

Moreover, the need to reduce the dependency on egg supplies for the manufacture of influenza vaccines has become evident during the last few decades: this is due to the growing demand for seasonal flu vaccines worldwide, together with the prospect of avian influenza viruses achieving sustained human transmission and concomitantly, to the occurrence of the A/H1N1v pandemic in the 2009 season.

Overall, therefore the need for better preventive strategies against influenza than those currently in use is clearly evident: the main critical issues and desiderata for influenza vaccines are outlined in **Table 1**.

Vaccines Currently Available and in the Last Stage of Clinical Development Against Influenza: Inactivated Adjuvanted and Intradermal Vaccines, Live Attenuated Cold-Adapted Intranasal Vaccines, Cell-Culture Formulations, Reverse Genetic-and DNA-Based Vaccines

To fill the gaps with conventional vaccines, several strategies have been investigated during the last few years, searching for innovative formulations able to offer a higher and broader immune response, or an equivalent response at a lower antigen dosage, while maintaining a good safety and acceptable tolerability profile. Some of these research approaches have led to the licensure of new influenza vaccines, both seasonal and pre-/pandemic, that have been adopted in several countries, while others are at an advanced stage of clinical development.

The use of adjuvants has represented one of the most successful means, during the last decade, in the attempt to a possible "gold standard" vaccine.[27] With the term "adjuvant," we refer to a large family of various biological and chemical compounds that, when added to a vaccine, are able to specifically enhance the immune response of the vaccine to the antigen presented, with a range of points, differing one substance from another. Some of the adjuvants used and investigated, in the context of influenza vaccines, are outlined in **Table 2**.

Since 1997, only two adjuvanted seasonal influenza vaccines have been widely used in several countries, with a million doses of both formulations being administered: a virosomal subunit vaccine (Inflexal V®, Berna-Crucell, Switzerland), for use in subjects aged 6 months or more, and a MF59-adjuvanted subunit vaccine (Fluad®, Novartis Vaccines, Italy), licensed for individuals aged ≥65 years.

Virosomes are reconstituted influenza virus envelopes devoid of inner core and genetic information. The influenza surface antigens, NA and HA, are integrated into phosphatidylcholine bilayer liposomes: by mimicking native viruses, the virosomes maintain the cell entry and membrane-fusion properties and act as an antigen delivery and presenting system, allowing presentation to the MHC class I and class II pathways.[28]

The main characteristics and results of the MF59-adjuvanted influenza vaccines are discussed below.

Both virosomal and MF59-adjuvanted vaccines have been widely investigated in several clinical trials, performed in populations of various ages (i.e., elderly, adults with various chronic conditions and young children) and in the post-marketing surveillance, showing good results in terms of tolerability and safety, as well as better immunogenicity than TIVs in the seasonal formulations, as recently reported elsewhere.[28–33]

From an analysis of the few comparative head-to-head immunogenicity studies published in the literature to date, no definitive conclusions can be drawn regarding which of the two adjuvanted vaccines is more immunogenic or effective in the elderly.[34-38]

Moreover, adjuvants have been used for their potential to significantly reduce the antigen dose in the vaccine formulation, while still maintaining a good immunogenicity profile: the so-called "dose-sparing" strategy.[39] Taking into account the length of time usually needed to manufacture the vaccine, this feature is of crucial importance, in terms of productive capacity and rapid availability for widespread human use, representing a fundamental tool in a pandemic emergency. Coinciding with the current influenza pandemic, caused by the new emergent A/H1N1 influenza viral strain, this characteristic has been demonstrated for the new MF59-adjuvanted A/H1N1 monovalent pandemic vaccine (Focetria®, Novartis Vaccines, Italy), which has shown optimal immunogenicity in healthy adults, at a 7.5 μg antigen dosage.[40,41]

The same is valid for another adjuvanted influenza vaccine, developed using an innovative adjuvant system, called AS03. This tocopherol oil-in-water emulsion-based adjuvant system has been tested, during the last few years, in a candidate H5N1 pre-pandemic influenza vaccine and has recently been adopted in the licensed formulation of a current A/H1N1 pandemic vaccine (Pandemrix®, Glaxo Smith Kline, Germany): good immunogenicity, together with an optimal safety and acceptable tolerability profile, have been demonstrated for this formulation in healthy adults, using a 3.75 μg antigen dosage.[42,43] Furthermore, the AS03-adjuvanted vaccine has been demonstrated to elicit a cross-reactive antibody response against heterologous viral challenge, in the same target population.[44] Nevertheless, the use of this product in the prevention of seasonal influenza is still under investigation.[45,46]

Another strategy that has led to the licensure of a new influenza vaccine is represented by the live attenuated influenza vaccines (LAIV) (Flumist®, Medimmune, USA). Unlike inactivated formulations, these products are constituted by cold-adapted viruses in which the HA and NA of the target strains are inserted and are administered by intranasal inoculation.[47] This vaccine, on the market, in the US since 2003, but not yet in Europe, is available for the immunization of healthy children and adults, aged between 2–49 years: data related to young children suggest that LAIV offers significantly greater efficacy than conventional vaccines, with values more than 80% for influenza A viruses and

Table 2. Adjuvants used and others under investigation in the context of influenza vaccines

Adjuvant category	Types
Oil-in-water emulsions	• MF59* • AS03* • AF03** • CoVaccine HT**
Saponins and glycolipids	• QS-21*** • ISCOMATRIX** • Alpha-GalCer (alpha-galactosylceramide)**
Liposomes	• Virosomes* • CCS (ceramide carbamoyl-spermine)** • CAF01 (cationic liposomes and synthetic mycobacterial cord factor)** • Vaxfectin**
Bacterial toxins/ components	• CT (Cholera toxin)** • LT (*Escherichia coli* labile enterotoxin)*** • Chitosan** • Salmonella and *Escherichia coli* flagellins**
Cytokines	• IL-12, IL-23, IL-28B** • GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor)** • Type 1 IFN (IFNalpha)**
TLR agonists/ immunomodulators	• Synthetic lipid A adjuvant (TLR-4)** • Bacterial flagellines (TLR-5)** • CpG (oligodeoxynucleotide) (TLR-9)*** • Polyl:polyC12U [(synthetic double-stranded RNA (dsRNA)] (TLR-3)** • IC31 (oligodeoxynucleotide) (TLR-9)** • sLAG-3 (IMP321) (ligand for MHC class II)***
Biomedical polymers	• PCPP (polyphosphazenes)**

*(in clinical use); **(investigated in animal model); ***(in clinical development).

more than 70% for influenza B viruses, even if it appeared to be less effective in healthy adults, when tested against drifted strains.[48-51]

This could be due to an over attenuation of the viruses in an immunologically not-naïve population.

More recently, another vaccine administered using a different route from the intramuscular (IM) injection, has been licensed in Europe: a new split trivalent intradermal (ID) vaccine (Intanza®, Sanofi Pasteur, France) has been developed using an innovative microinjection system (Soluvia®, Beckton Dickinson, USA): two ID formulations are currently available on the market, one for adults aged 18–59 years (9 μg/HA strain) and the other for the elderly ≥60 years (15 μg/HA strain).[52] Details concerning the immunological mechanisms and the rationale of the ID route for the administration of preventive vaccines, together with the more recent findings concerning the safety and immunological profile of this new product, are outlined below.

Cell culture-based technology is also particularly suitable for the manufacture of influenza vaccines, with the WHO recommending use of established mammalian cell culture lines as an alternative to egg-based substrates for this purpose.[53,54] Undoubtedly, cell culture manufacturing is one of the major innovations in influenza vaccine production, during the last few decades, being used for the development both of inactivated and live attenuated formulations.[53-56] Currently, vaccines produced in three different host cell lines (Madin-Darby Canine Kidney—MDCK, Vero and PER.C6) have been developed and investigated in clinical trials.[53] This approach has led to the licensure of both pandemic vaccines against A/H5N1 and A/H1N1 strains (Celvapan®, Baxter, USA). In 2007, an influenza inactivated vaccine, developed with MDCK cell culture, has also been approved by the European Union for use in adults (Optaflu®, Novartis Vaccines, USA). Together with the increased and rapid capacity of the vaccine production, another benefit of the cell culture approach is that these products can be safetly administered to allergic subjects. The increased knowledge regarding cell culture systems promises significant improvements leading to the rapid development of preventive vaccines against a wide range of viral diseases besides flu.

Another important goal recently achieved in the preparation processes of influenza vaccines has been the use of plasmid based reverse genetic systems, which are able to obtain a specific genetic composition of the seed strains, thus allowing more rapid generation of the viruses to be included in the vaccine composition by the WHO, resulting in positive implications not only for seasonal vaccines but particularly for those against highly pathogenic pandemic strains.[27,57-60]

DNA vaccines, offering the potential to elicit a robust and broad-spectrum humoral and cellular immunity, have also been explored, both in animals and humans, for the prevention of a wide variety of diseases: as far as concerns influenza, this technology has been studied for the development of vaccines against avian H5N1 and H9N2 strains.[61-64] These products promise to be a better preventive tool to more effectively face the threat of an influenza epidemic or pandemic from the public health viewpoint, thanks also to their relatively safe, cheap and fast manufacturing process of production. Albeit, clinical studies are needed to confirm the positive results already reported, in the animal models, in terms of safety, immunogenicity and efficacy.

An overview of the most recent and promising results, part of which emerging from our personal experience, concerning two successful approaches of those previously outlined, namely the use of: (i) the oil-in-water MF59-adjuvant and (ii) the ID route of administration, are reported and discussed below, together with the possible implications and future perspectives aimed to optimize immunization policies against influenza during the next few decades.

MF59-Adjuvanted Vaccine: A Safe and Useful Tool to Enhance and Broaden Protection Against Influenza Viruses

The MF59-adjuvanted influenza vaccine is a mixture of influenza antigens and a low oil-in-water emulsion of squalene, a naturally occurring biodegradable and biocompatible substance, found in the liver in a wide range of species, including humans.[31]

MF59 emulsion induces a local immune-stimulatory environment, which is able to optimally activate the innate immune response at the injection site, by recruiting and activating antigen-presenting cells, which are then better able to capture, transport and process co-administered antigens from the peripheral tissues to local lymph nodes and consequently, stimulate an effective adaptive memory immune response specific to the vaccine.[65-67] The detailed mechanisms involved in the immune response, following immunization with the MF59 adjuvant, have been described in a recent review from our group.[30]

Following an extensive clinical development, the MF59-adjuvanted seasonal influenza vaccine (Fluad®, Novartis Vaccines, Italy) was first licensed, in Italy, in 1997. It is currently available in several European and non-European Countries and more than 45 million doses have been distributed so far.[68] The specific therapeutic indication of the MF59-adjuvanted seasonal influenza vaccine is active and routine immunization of the subjects aged ≥65 years.

In September 2009, as previously mentioned, a MF59-adjuvanted monovalent A/H1N1 pandemic influenza subunit vaccine (Focetria®, Novartis Vaccines, Italy) was authorized for human use by the European Medicines Agency (EMA), with approved therapeutic indications for active prophylaxis against pandemic influenza in infants and young children (aged ≥6 months), adults and elderly subjects.[69] The most recent data, available on 17 April 2010, indicated that at least 36 million doses of Focetria® had been distributed in the European Economic Area and at least 6.5 million patients had been vaccinated with this new formulation.[70]

The MF59-adjuvanted influenza vaccines have been demonstrated to be safe and well tolerated, as reported both in phase I-IV clinical studies, performed in nearly 14,000 individuals, mostly elderly, but also in adults, adolescents and children and in post-marketing pharmacovigilance data.[30,32,33,65] In particular, tolerability results were shown to be good, also after consecutive repeated immunizations, with no significant trend for increased

reactogenicity:[71] generally, when compared to non-adjuvanted vaccines, MF59-formulation is associated with higher rates of local side-effects, particularly pain, followed by erythema and induration at the injection site, but these reactions are mostly transient and mild. Systemic side-effects, such as myalgia, general malaise, headache and fever, are not usually frequent.[71] Analyses of surveillance data, collected from September 1997 to August 2006, in more than 27 million individuals, mainly elderly, receiving the MF59-adjuvanted influenza vaccine, reported no fatal cases causally related to the administration of the vaccine and furthermore reported a frequency of serious adverse reactions, following immunization, of 1.4 cases per 100,000 doses: this rate is not greater than the expected spontaneous incidence in the general population and, notably, it is lower than that recorded for influenza immunization by other surveillance systems outside Europe, in Countries using conventional vaccines (i.e., in Australia 1.8–2.1 per 100,000 doses).[33] Furthermore, a recent analysis from the database of clinical trials, regarding the risks associated with exposure to MF59-adjuvanted influenza vaccines during pregnancy, demonstrated that the distribution of pregnancy outcomes was similar between mothers exposed to MF59-adjuvanted and to unadjuvanted influenza vaccines, at any time during pregnancy, although data were too few to draw definitive conclusions.[72] According to a recent pharmacovigilance update by the EMA, also the safety profile of Focetria can be considered similar to that of the MF59-adjuvanted seasonal influenza vaccine.[70]

As far as concerns the immunological profile of the MF59-adjuvanted influenza vaccine, a number of clinical trials have demonstrated that it enhances immunogenicity, in terms of antibody titers, seroconversion and seroprotection rates, in the elderly, living either in institutionalized or outpatient settings, when compared to conventional non-adjuvanted vaccines:[35,38,71,73,74] this positive effect has been confirmed in subjects following subsequent immunizations during consecutive seasons[75] and in subjects with a low titer of specific antibodies prior to immunization.[31] The capacity of the MF59-adjuvanted seasonal influenza vaccine to enhance the immune response, compared to conventional vaccines, was also demonstrated in the elderly and adult patients affected by chronic underlying medical conditions, such as respiratory or cardiovascular diseases, cancer and metabolic disorders.[38,74,76,77] This positive enhanced immunogenicity has also been reported in other populations at high risk of influenza, namely renal transplant recipients and Human Immunodeficiency Virus (HIV)-infected individuals, in whom the vaccination did not show any negative effect upon the natural clinical course of the disease (no changes in viremia and $CD4^+$ cell-count post-immunization).[78-81]

The MF59-adjuvanted seasonal influenza vaccine has also recently been shown, for the first time, to enhance the immunogenicity, compared to conventional vaccines, in healthy children, both unprimed aged <3 years and primed from 16 to <48 months of age.[82,83]

Interestingly, the MF59-adjuvanted seasonal influenza vaccine has been reproducibly demonstrated to confer cross-reactivity against drifted influenza virus strains in the elderly,[17,18,38,84] in adults with serious underlying medical conditions,[23,85] and, recently, in healthy unprimed young children:[82] the enhanced immunogenicity against heterovariant strains represents, to date, a unique property of this influenza vaccine, compared to other seasonal formulations currently available.

In this context, our research group assessed the immune response of elderly subjects, immunized with MF59-adjuvanted or non-adjuvanted subunit influenza vaccines, against a A/H3N2 vaccine strain (A/Wyoming/3/03), three egg-passage reference vaccine candidates (A/California/7/04, A/Wisconsin/67/2005 and A/Brisbane/10/07) and three drifted isolates, phylogenetically close to the above-mentioned vaccine viruses (A/Genoa/13/04, A/Genoa/2/05 and A/Genoa/7/08), by using both the hemagglutination inhibition (HI) assay, at present considered as the "gold standard" test for the evaluation of vaccine-induced antibody response, and neutralization (NT) assay, that may provide a more functional assessment of vaccine-induced immunity, thus being more sensitive than HI. Both vaccines met the seroprotection and mean-fold-increase requirements of the Committee for Medicinal Products for Human Use (CHMP), against the vaccine strain, whereas a substantial response against strains not included in the vaccine composition, was observed only in individuals immunized with the MF59-adjuvanted vaccine. The results, consistent with other findings from our research group, collected over the last decade, confirm that the MF59-adjuvanted vaccine elicits a stronger immune response than non-adjuvanted vaccines against homologous strains. When the immune response was evaluated against drifted strains, however, the immunogenicity profile of the two vaccines differed considerably,[18] being higher in subjects immunized with the MF59-adjuvanted vaccine.

More recently, we compared the antibody response, using HI and NT assays, elicited by MF59-adjuvanted and non-adjuvanted subunit vaccines containing the A/H3N2/California/7/04 strain against circulating viruses isolated between 2004/2005 and 2006/2007 seasons, belonging to A/H3N2/California/7/04 and presenting amino acid mutations onto antigenic sites with respect to the vaccine virus with "apparent" good antigenic matching. The main results (**Fig. 1**) demonstrated that the advantage offered by MF59 adjuvant in terms of higher immunogenicity, expressed as higher post-vaccination HI-titers, is found also against viruses showing antigenic and molecular patterns undistinguishable from the vaccine strain, but this became even more evident as the antigenic and molecular distance between vaccine and circulating strains increased.[84]

By contrast, the recent evaluation of the protection offered by the MF59-adjuvanted subunit influenza vaccine, for the 2003/04 winter season, containing an influenza B/Victoria-like antigen B (B/Hong Kong/330/01) against mismatched and frequently co-circulating variants of influenza B/Victoria- and B/Yamagata-like virus strains, showed that the immunization induced significant increases in the amount of HI antibodies, in middle-aged and elderly subjects, against all influenza B strains under investigation, including the heterologous strains, but the response against the heterologous B/Shanghai/361/02 virus did not meet the requirements of the European Commission in either of the age-populations: these data would support the recommendation of

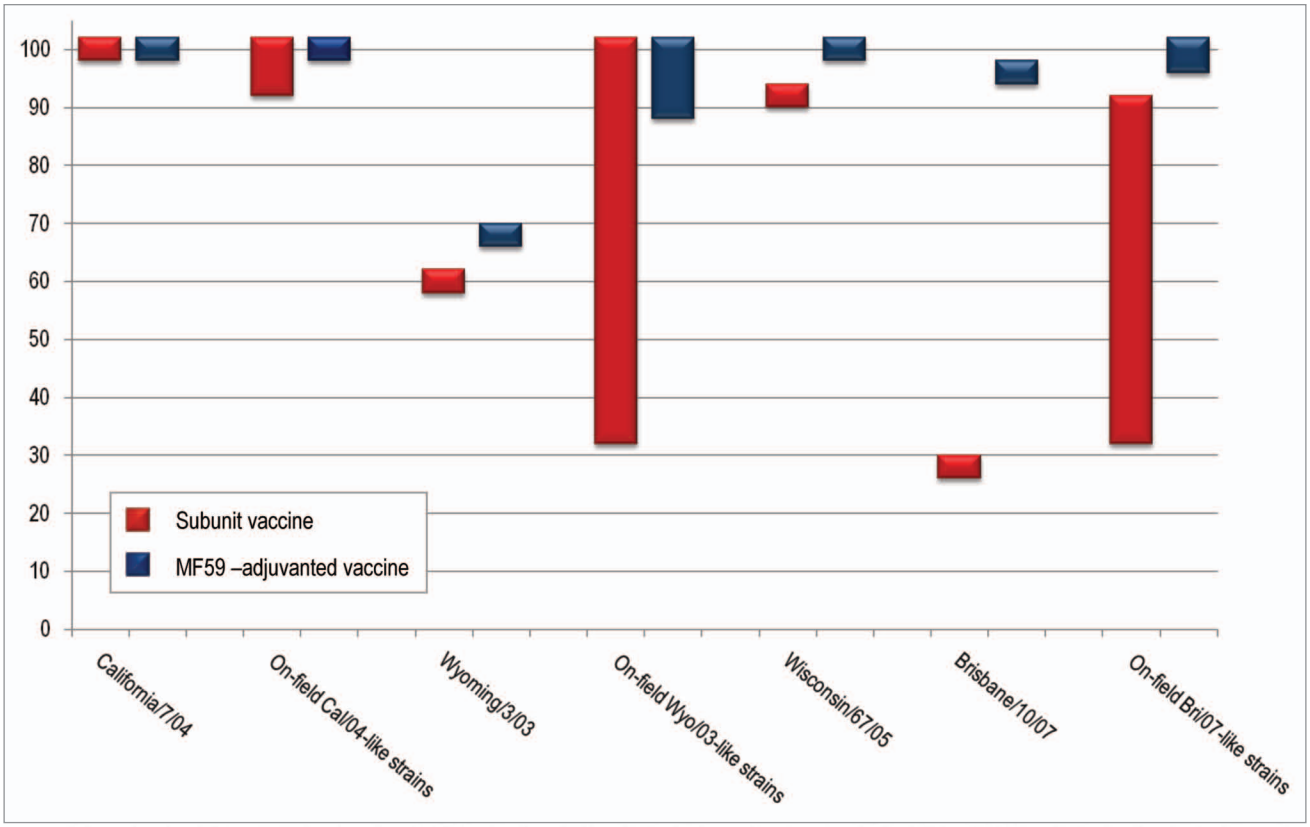

Figure 1. Comparison of the seroprotection rate (%) ranges, determined by hemagglutination inhibition (HI) assay, against the vaccine strain A/California/7/04, following vaccination with a MF59-adjuvanted vaccine and non-adjuvanted vaccine, according to several viral strains different from that included in the vaccine.

including influenza B viruses of the B/Victoria and B/Yamagata lineages in future influenza vaccine preparations.[86]

The capacity of the MF59 adjuvant to offer enhanced immunogenicity against heterovariant strains has also been well documented by the MF59-adjuvanted A/H5 influenza pre-pandemic vaccines, both in elderly and non-elderly adults.[87,88] Moreover, recent findings demonstrated that two 7.5 μg doses of the MF59-adjuvanted vaccine against A/Vietnam/1194/2004, administered in subjects primed, at least six years earlier, by an antigenically distinct MF59-adjuvanted vaccine, induced a rapidly mobilized and long-lasting immune memory,[89] mediated by a pool of cross-reactive memory B cells, that can be rapidly boosted years afterwards by a mismatched MF59-adjuvanted vaccine, to generate high titers of cross-reactive neutralizing antibodies.[90] Another recent study, evaluating the immunogenicity of a single booster dose of an MF59-adjuvanted H5N1 vaccine, containing 7.5 μg A/turkey/Turkey/1/2005-like H5N1 HA, given approximately 18 months after primary vaccination with a heterologous strain, confirmed that the booster vaccine induced a robust and cross-reactive immune response.[91] Pre-pandemic vaccination could thus be considered as a proactive vaccine-priming strategy, representing a useful tool, particularly among those categories at high risk of pandemic influenza, in order to rapidly generate cross-clade antibodies, even after a single vaccination or after exposure to the pandemic virus.

Another important aspect of the use of adjuvants, investigated in clinical trials assessing the immunogenicity of pre-pandemic and pandemic influenza vaccines, is their potential for reducing the antigen dose in the vaccine formulation, while still maintaining a good immunogenicity profile: the so-called "dose-sparing" strategy.[39] At the same time as the recent A/H1N1 influenza pandemic, this characteristic has been demonstrated for the new MF59-adjuvanted A/H1N1 monovalent pandemic vaccine, which showed optimal immunogenicity in healthy adults, at a 7.5 μg antigen dosage.[40] A multicenter clinical trial, including our Center, recently performed in Italy on healthy adults, aged over 18 years, aimed to evaluate the immunogenicity of the novel A/H1N1v vaccine in subjects already vaccinated with seasonal influenza vaccines. Results showed that one dose of MF59-adjuvanted A/H1N1 monovalent vaccine, at a 7.5 μg antigen dosage, or even at the lower dose of 3.75 μg, with a half dose of MF59, provides protection for the majority of adults and elderly subjects, meeting the CHMP criteria for pandemic influenza vaccine licensure.[41]

Although an exact evaluation of the effectiveness, offered by the influenza vaccines, is difficult to establish, some useful data concerning the clinical impact of the MF59-adjuvanted vaccine are available in the elderly. In particular, the effectiveness of the MF59-adjuvanted influenza vaccine has been demonstrated in older adults, thus avoiding emergency admissions for pneumonia

and hospitalizations for pneumonia, cardiovascular and cerebrovascular diseases.[92,93]

The only on-field study directly comparing the effectiveness of the MF59-adjuvanted influenza vaccine and of a non-adjuvanted subunit formulation, performed in almost 2000 elderly residents, in long-term care facilities, in northern Italy, recently demonstrated that the risk of acquiring influenza was superior for the non-adjuvanted vaccine recipients, compared with the MF59-group [Odds Ratio (OR) = 1.52, 95% Confidence Intervals (CI) = 1.22–1.88] and was highest for those affected by respiratory diseases (OR = 2.27, 95% CI = 1.09–4.82) and cardiovascular diseases (OR = 1.88, 95% CI = 1.31–2.72).[94]

In conclusion, the MF59-adjuvanted seasonal influenza vaccine has reproducibly been shown to enhance immunogenicity and to confer cross-reactivity against heterologous viral strains compared to conventional non-adjuvanted vaccines in the elderly. The same features have also been demonstrated with the MF59-adjuvanted pre-pandemic and pandemic vaccines.

An enhanced and broader immune response was also demonstrated in the elderly and in adults presenting a wide range of serious underlying medical conditions, recently, also in infants and young children.

MF59-adjuvanted influenza vaccines are safe, as demonstrated in several clinical trials, and by robust data emerging from post-marketing pharmacovigilance.

The Intradermal Influenza Vaccines: Rationale and Clinical Experience

Advances in the field of immunology have led to a better understanding of the dense network of immune-stimulatory cells present in the epidermis and dermis, such as Langerhans cells, macrophages, mast cells, Dendritic Cells (DCs) and leukocytes, promoting the use of innovative transcutaneous routes for administration of vaccines also thanks to the development of new injection techniques. The recent availability of innovative microinjection systems has led to a renewed interest in the ID administration of flu vaccines, not only for the easy access to the skin but also due to the particular immunological characteristics of this organ.

In more detail, the rationale for the ID route lies in the demonstrated ability of the resident DCs to amply stimulate the innate immunity, thus increasing the adaptive immune response to immunization, as well described in a recent review by Nicolas and Guy.[95] This is also favored by the thick network of the microvascular dermal unit, located in the papillary dermis, near the dermal-epidermal junction, allowing an ample exchange between the skin and the blood and lymphatic system, thus facilitating the antigen presentation in the lymph node.

Once vaccine is delivered by the ID route, immature DCs, residing in the papillary dermis, capture and process the antigen and subsequently, re-express some of its peptides in the groove of the MHC class I/II on the cell surface. These peptides, together with the non-self danger signal triggered by the injection, induce maturation of the DCs and their migration to the regional lymph nodes through the afferent lymphatic vessels. This mechanism is activated and facilitated by pro-inflammatory cytokines, particularly IL-1β and TNFα, that play a key role in the migration of DCs to the paracortical area of the regional lymph nodes, where they act as antigen presenting cells.[95] Moreover, during the migration process through the draining lymphatics, DCs undergo functional maturation, losing the ability to process antigen, but acquiring immune stimulatory properties aimed at recognition by naïve T-cell receptors and specific precursor B cells.[96]

In the paracortical area of the lymph node, the complex peptide-MHC class I and peptide-MHC class II are specifically recognized by $CD8^+$ T cells and $CD4^+$ lymphocytes, respectively. $CD4^+$ T cells promote the differentiation of B cells into plasma cells able to produce and release antibodies into the systemic circulation, while $CD8^+$ T-cell precursors proliferate clonally and enter the circulation through the efferent lymphatic vessels and the thoracic duct, acquiring skin-specific homing antigens (CLA and CCR4) and becoming effectors and memory T cells.[95]

Recent studies have also shown that ID administration of the antigen also improves the recruitment of DC precursors from the blood into the dermis, and their subsequent migration to the lymph node: this is an important issue for the priming and differentiation of T cells, particularly $CD8^+$ T cells, into effector cells.[97]

Furthermore, the antigen delivered via the ID route can reach up free the regional lymph nodes through the lymphatics: here, it is able to activate directly, through interaction with the B-cell receptor (BCR), specific B-cell precursors. Following internalization and processing of the BCR-antigen complex, the antigenic epitopes are expressed on the surface of B cells through the complex peptide-MHC class II. Therefore, B cells present the antigen to $CD4^+$ T cells, which induce the differentiation of the B cells into antibody secreting plasma cells.[95]

The complex mechanisms activated by ID immunization, together with those elicited by IM vaccination, are illustrated in **Figure 2**. Clearly, the immune response induced by ID antigen delivery is generated by a mechanism which markedly differs from that of the IM route: in the deep muscle, only circulating DCs are able to capture the antigen and to migrate to lymph nodes through lymphatic drainage or general circulation.

Despite the immunological advantages described above, ID vaccine administration has met several difficulties, in clinical application, due to the injection techniques used so far, such as the Mantoux technique, for the tuberculosis skin test and the bifurcated needle, employed for polio vaccine.[98]

In recent years, particularly thanks to the development of an innovative microinjection system (Soluvia®, Beckton Dickinson, USA), the ID route has been widely investigated in clinical trials of influenza vaccine resulting in easier use, and being more reliable and safer with respect to the traditional IM injection.[99-103] This new microinjection system, currently the only intradermal device licensed for influenza vaccines, has a micro needle approximately 1.5 mm in length, integrated with a pre-filled syringe ready for use that has a system specifically designed to limit the depth of penetration and injection to ensure proper placement of perpendicular needle insertion, reducing blood vessel and nerve injuries in patients. The syringe also has an automatic needle shielding system that is activated following completion of the injection,

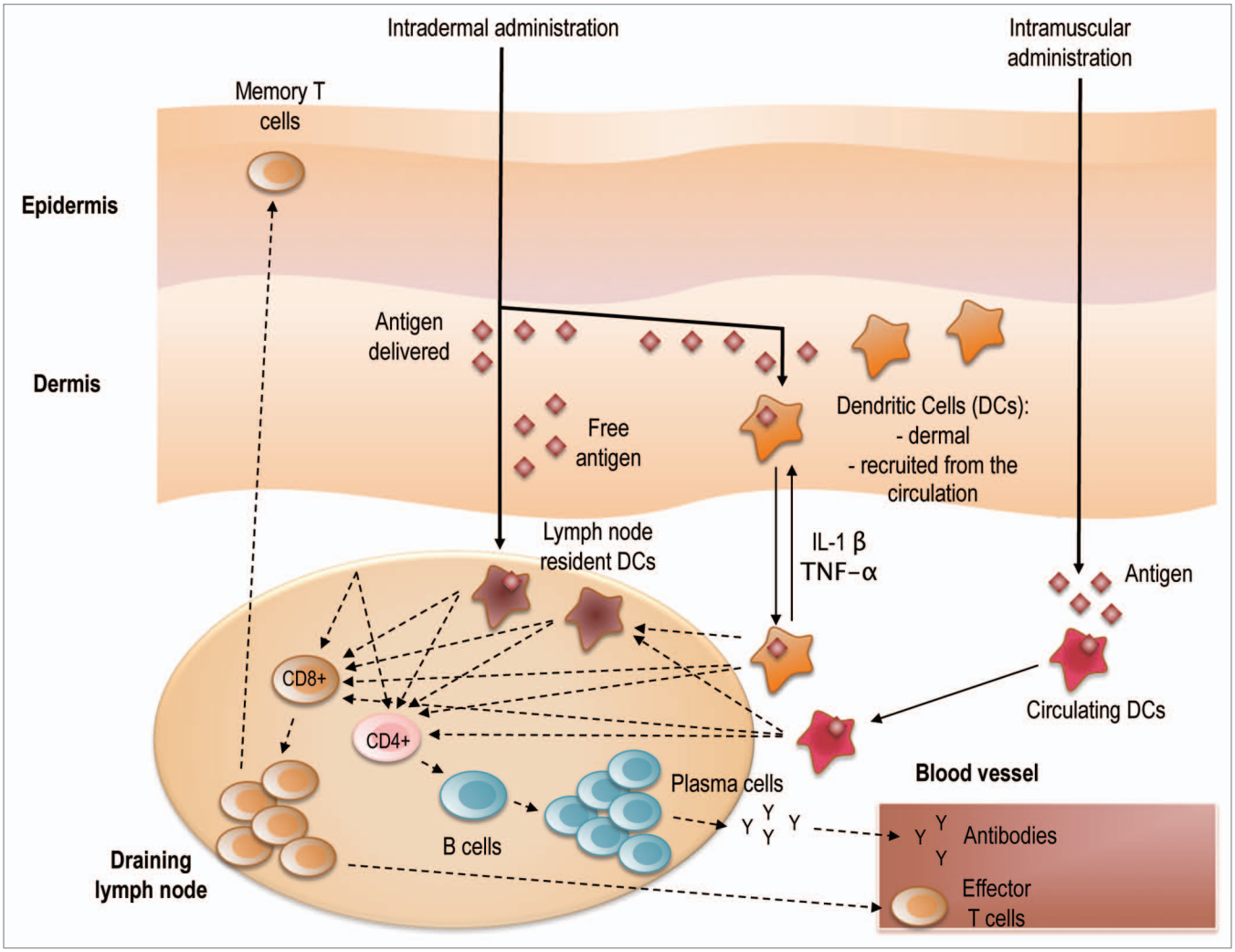

Figure 2. Mechanisms and cells involved in the innate and adaptive immune response following administration of a vaccine antigen using the intradermal and intramuscular route. Adapted from reference 95.

thus reducing the risk of accidental puncture for healthcare workers and also preventing re-use of the device.[104]

In addition to these practical advantages, numerous clinical trials have focused on the immunogenicity of the ID seasonal influenza vaccines, particularly on two main objectives: (i) reduction of the antigen content to achieve the same immunogenicity as the standard vaccine (dose-sparing) and (ii) improvement of the immune response, maintaining the same dosage as the standard intramuscular vaccine. Results collected, during the last few years, have demonstrated that ID influenza vaccines are able to confer a better immune response than TIVs at a full antigen dosage in the elderly and an equivalent response, at a lower antigen dose, in healthy adults and in patients with severe chronic diseases or immunocompromized.

Several studies, performed on healthy adults to evaluate the dose-sparing strategy, demonstrated that non-adjuvanted vaccines at different antigenic concentrations (3, 6 and 9 μg/HA virus strain), administered using ID route, were able to elicit immune responses equivalent to those obtained with full dose IM vaccines or, at least, to meet the criteria of CHMP for the licensure of influenza vaccine.[101,102,105-110]

In particular, two recent clinical trials, comparing a 9 μg/HA strain trivalent split ID vaccine, using the novel microinjection system Soluvia®, with a traditional IM formulation, in a population of over 2000 healthy adults, were crucial for the licensure of the ID seasonal influenza vaccine in Europe (Intanza®, Sanofi Pasteur, France).[101,102]

The first trial, a phase II, multicenter, randomized open-label study, by Leroux-Roels et al , performed in subjects receiving a 0.1 mL injection of ID trivalent vaccine, containing 9 μg/HA strain (n = 588), or a conventional 0.5 mL intramuscular vaccine (15 μg/HA strain; n = 390), showed that the ID trivalent inactivated influenza vaccine induced a superior humoral immune response against both A strains (H1N1, H3N2), and a non-inferior immunogenicity against B strain, compared with the conventional IM vaccine, offering a good safety and tolerability profile.[101]

The second study, a phase II, multicenter, randomized, partially blinded, controlled study, investigated the immunogenicity

and safety of three different dosages (3, 6 and 9 μg/HA strain) of the trivalent, inactivated, split-virion ID vaccine against seasonal flu with an IM control vaccine (15 μg/HA strain), during 3 consecutive seasons.[102] The 3 μg and 6 μg ID formulations were less immunogenic than the IM full dose, and non-inferiority was not demonstrated, while the 9 μg ID formulation, administered during the second and the third seasons, was found to be comparably immunogenic to the control vaccine, satisfying the EMA criteria for all three virus strains. The reactogenicity of the ID vaccine was comparable to that of the IM vaccine, and local inflammation was found to be more frequent following the ID vaccination than the IM administration: however, these reactions were not associated with an increased incidence of pain at the injection site.[102]

As previously mentioned, ID administration has been studied also in view of improving the immunogenicity of seasonal influenza vaccines in some at risk target groups of the population.

For this purpose, the ID strategy has been investigated in the elderly and, recently, two conclusive studies on the ID seasonal influenza vaccine, administered using the new microinjection system Soluvia®, have been published.

Holland et al. were the first to demonstrate, in a phase II, multicenter, randomized clinical trial on over 1,000 subjects, aged >60 years, that influenza ID vaccine, containing 15 μg/HA virus strain, elicited an immune response significantly superior, in terms of seroconversion, seroprotection and mean titer increases, except for seroprotection against A/H1N1, to that achieved using conventional split IM vaccination.[99] The ID immunization induced an immune response that satisfied, not only the EMA requirements defined for the elderly but, also, the higher values required for younger adults,[111] thus opening new important perspectives for the control of influenza in this particularly vulnerable category.

The other study, including also our Center, further confirmed the benefit of the ID vs. IM vaccine for immunization against seasonal influenza in the elderly.[100] This phase III multi-center, randomized, open-label clinical trial, was performed in over 3,707 subjects, aged ≥60 years, during three consecutive seasons. The subjects enrolled were randomized to receive two vaccines, ID or IM, both containing 15 μg/HA per strain: an in-depth analysis of the safety, tolerability and immunogenicity of the two formulations was performed at the end of the study period. During the first year, both vaccines met the CHMP immunogenicity criteria for older adults for both Influenza A strains and two out of three for the B strain,[111] the ID vaccine offering significantly higher seroprotection rates and inhibition antibody titers, for all three strains, compared to the IM vaccine. After the second and third annual vaccinations, the superior immune response of the ID vaccine was maintained, as demonstrated by seroprotection rates. Moreover, a good safety profile, recorded in terms of occurrence of serious adverse events, was observed during the entire study period. The tolerability profile, measured by incidence of systemic side-effects, was similar in the two treatment groups, while in patients immunized with the ID vaccine a greater local reactogenicity was observed. The extent of local effects was mostly mild and of short duration (less than 1% of the subjects reported a reaction of >3 days).[100]

Very recently, the first head-to-head study comparing the new ID vaccine to the MF59-adjuvanted formulation, in the elderly, has been published.[112] This phase III trial was carried out during the influenza season 2007–2008 on a population of 795 adults, aged ≥65 years, randomized to receive one of two vaccines, each containing 15 μg of HA per virus strain. Non-inferiority of the ID vaccine was demonstrated for all three virus strains by the single radial hemolysis (SRH) method and for A/H1N1 and B strains using the HI test. Both vaccines satisfied the EMA immunogenicity and safety criteria for influenza vaccines, as established for the elderly. As far as concerns tolerability, local reactions were earlier and more common following the ID administration than after IM injection, but signs and symptoms were mild and of short duration, lasting <3 days, and were not associated with a higher incidence or severity of injection-site pain. This clinical observation results from the pro-inflammatory environment created following direct injection of the vaccine content in the dermis, just below the skin surface.

Concomitantly with the encouraging findings in healthy adults and the elderly, positive results in terms of either dose-sparing or improvement of the immune response, with respect to TIVs, have been obtained using the ID strategy for influenza vaccination of patients with severe chronic diseases or immunocompromized (i.e., patients with solid cancer, patients treated with anti-TNFα, persons infected with HIV, patients who have undergone haematological stem cell transplantation as well as renal transplant recipients).[113-115] Nevertheless, in a study performed in chronic obstructive pulmonary disease patients, who received either 0.2 ml (6 μg of HA per virus strain) split into two site ID injections or a single 0.5 ml, full dose, IM injection, antibody responses of the ID arm were lower than those of IM group, even if each strain of the ID vaccination met the CHMP requirements.[116]

In conclusion, based upon the reported clinical experience, ID vaccines need to be considered safe and immunogenic, being a valid alternative to other currently available products for the active immune-prevention of seasonal influenza in both adults and elderly individuals. Moreover, they represent a new important tool from the viewpoint of public health, offering the possibility of dose-sparing. The less invasive route of administration, on the one hand, together with the simple, rapid, reproducible and safe technique of inoculation, on the other, are additional factors which could increase compliance and acceptability to be vaccinated.

Further studies are needed in patients affected with severe chronic diseases and immunocompromized, in order to confirm the benefit, in terms of protection, of the ID vaccines vs. conventional vaccines. Moreover, few safety and immunogenicity studies have been performed in infants and young children to date: additional data are needed to evaluate the potential of the ID strategy in this setting.

Looking at the recent history of the field of immune-prevention, ID route can be considered an attractive approach for the administration of vaccines, also thanks to the development of new delivering technology, and could open interesting perspectives in order to improve the prevention, not only of influenza, but also of other infectious diseases in the near future.

Future Perspectives

There is no doubt that significant progress has been made, during the past decade, in the field of the prevention of influenza, thanks also to the development and licensure of new safe and more immunogenic vaccines than the conventional types. In our experience, MF59-adjuvanted vaccines, together with ID vaccines, can certainly be considered as successful examples of this improvement.

Nevertheless, it should be stressed that, as yet, there is no vaccine that can be considered as "ideal" for the optimal control of influenza, either during interpandemic periods, or even more, in the event of a pandemic caused by a highly pathogenic flu strain. Thus, in short to midterm period, hopefully we can look forward not only to improvements in rapid vaccine manufacturing technologies (i.e., cell culture systems, reverse genetic- and DNA-based vaccines) but also to the introduction, in the clinical field, of the use of innovative products conferring more cross reactivity and more efficacy than those offered with the currently available formulations.

Together with this challenge, standardization of the serological tests to be used in the assessment of the humoral response, together with the introduction of laboratory-analyses focusing on the T cell-mediated immune aspect, should be carefully taken into consideration by the regulatory gencies for the evaluation and licensure of flu vaccines, with necessary updating of the immunological parameters and criteria required for this purpose. Moreover, careful evaluation of the effectiveness of innovative vaccines, to be assessed in large and well-designed on-field clinical studies, will be mandatory in order to orientate public health immunization policies against influenza using an evidence-based approach in the near future: this also applies to currently available adjuvanted flu vaccines, for which data on this aspect are still lacking.

Ideally, the development of influenza vaccines that would protect for more than a few years and, hopefully, life-time, against any type/subtype of the various strains circulating in the epidemiological scenario, is the dream of all vaccinologists engaged in the control of influenza. From this viewpoint, the most promising approach is that regarding the production of vaccines based on more conserved antigenic epitopes than the highly variable surfaces of the HA and NA proteins, such as the extracellular portion of the M2 protein, the nucleoprotein and some conserved domains of the HA.[27,117-119] Positive preliminary results for a "universal target" antigen vaccine against influenza have been achieved using the extracellular portion of the M2 protein in the mouse model, in which antibodies elicited by immunization, directed to this domain, have been shown to confer protection against a range of influenza strains:[120] the magnitude of this immune response, in other animal models, remains to be further investigated and the same will be assessed in ongoing clinical trials, with many key-points of this very promising immunization strategy becoming clearer in the near future.

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