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NATURAL POLYMER X (NPX):

A NOVEL ADJUVANT FOR PROTEIN-BASED VACCINATION STRATEGIES

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Summary

The emerging popularity of subunit vaccines that are constituted by poorly immunogenic synthetic and recombinant antigens, has underlined the importance of discovering new and potent adjuvants able to modulate, enhance, and extend the immune response against these antigens. Many different classes of adjuvants are currently under development in pre-clinical or clinical stage, but only a few are licensed for clinical use. The class of natural polymers (NPs) is emerging as a promising alternative among adjuvant formulations; thanks to their chemical and physical properties and their potential for functionalization, natural polymers can be rationally modified to improve their adjuvanticity and to elicit an optimal immune response, making them attractive candidates for substituting conventional adjuvants. Natural polymer X (NPX) is a biodegradable, biocompatible, non-toxic, and non-inflammatory linear polysaccharide, which can be easily conjugated to virtually all types of antigens. Moreover, low molecular weight (LMW) NPX derivatives behave as a damage-associated molecular pattern (DAMP) molecules, acting as Toll-Like Receptor (TLR) agonists.

This project aimed at exploring the potentiality of NPX as an effective and safe adjuvant for protein-based vaccination studies. To this end, the ovalbumin (OVA) model antigen was chemically linked to NPX, and the resulting NPX-OVA bioconjugate was used for mice immunization. Different vaccination schedules, concentrations of the conjugated antigen, and molecular weights of NPX were tested, and induction of humoral response was evaluated by ELISA. Overall, results showed that NPX conjugation to the model antigen is able to strongly increase OVA immunogenicity, exerting a remarkable adjuvant effect. Indeed, our data demonstrated the superiority of NPX adjuvant over Alum, since NPX is efficient in inducing potent and long-lasting humoral responses that, differently from those obtained with Alum, are characterized also by the production of IgG_{2a} and IgG_{2b} subclasses. NPX conjugation to OVA was also shown to allow antigen and dose sparing, since OVA-specific IgGs were detected already after a single injection of NPX-OVA and also following immunization with very low antigen doses. Moreover, NPX was compared with other commercial adjuvants (Quil-A, Addavax, Complete/Incomplete Freund's Adjuvant (CFA/IFA), Chitosan, LPS, and Montanide), showing to induce an IgG production that is comparable or even superior to that produced by all the tested adjuvants. Only CFA/IFA-induced humoral response was higher than that generated with NPX-OVA, but NPX was always more efficient than other adjuvants already approved for clinical use (e.g. Alum, Addavax).

Experiments aimed at dissecting the capability of NPX of inducing a cellular immune response showed controversial results. Although immunization of mice with NPX-OVA proved to induce cytotoxic responses that were comparable to those induced by well-known Th1-inducing adjuvants, no significantly higher numbers of SIINFEKL-specific $CD8^+$ cells were detected by pentamer staining in different compartments following NPX-OVA immunization. Thus, the question whether NPX is also able to promote cytotoxic immune responses, or whether its adjuvanticity prevalently reside on its ability to generate a very potent and long-lasting humoral response, remains still open. In this work, another remarkable feature of NPX as adjuvant was highlighted: its biocompatibility at injection site and its generally safe profile. Results obtained by cell recruitment and cytokines analysis in muscles overall indicate that NPX is extremely safe and well tolerated when injected i.m., apparently acting differently from classical immunological adjuvants that trigger strong inflammatory responses at the site of injection. *In vivo* biodistribution studies confirmed by fluorescence microscopy analysis, indicated that NPX promotes the rapid migration of the antigen to the draining lymph node, thus inducing the accumulation of the antigen where the encounter with APCs is facilitated. In parallel, experiments performed *in vitro* confirmed that NPX-OVA can be digested by the enzyme NPXase, leading to the production of LMW fragments of NPX that are described in literature to act as TLRs agonists, and induce DCs maturation. This was confirmed by *in vitro* experiments, which demonstrated that stimulation with LMW NPX derivatives is able to activate bone marrow-derived dendritic cells (BMDCs). Thus, with regards of NPX mechanism of action, we hypothesize a potential scenario where NPX is responsible for the rapid drainage of the antigen to the lymph node, where it is preferentially accumulated. Simultaneously, NPX may undergo degradation by NPXase enzymes present in tissues. LMW NPX products generated from NPX digestion may function as DAMPs activating DCs trough binding specific TLRs and thus stimulating antigen recognition and presentation to effector cells.

Taken together, this work proposes NPX as a new and promising candidate adjuvant, offering insight into its major adjuvant features and its mechanism of action, and demonstrating its efficacy and tolerability in preclinical animal models. NPX was indeed shown to be extremely well biotolerated *in vivo*, and to induce robust and long-lasting humoral immune responses, leading to improved results as compared to other clinical-grade adjuvants; moreover, it was efficient with strongly reduced antigen concentrations and number of injections. In summary, our data demonstrate that NPX is a powerful and safe natural adjuvant, which could be exploited for the design of new and efficient protein-based vaccine formulations.

Introduction

1. The immune system against infectious diseases

The body's ability to fight pathogens or other foreign agents and to prevent infectious diseases, is defined as immunity. A fundamental concept of immunology is the classification of the immune system in two arms called innate and adaptive immunity. Innate immunity is genetically inherited and protects the individual from birth throughout life, acting as a first line of defense against pathogens. The main components of the innate immune system are physical epithelial barriers, phagocytes, dendritic cells (DCs), natural killer (NK) cells, and circulating plasma proteins. Acquired or adaptive immunity is constituted by components that are normally silent; however, when activated, these components "adapt" to the presence of infectious agents by activating, proliferating, and creating potent mechanisms for neutralizing or eliminating the microbes. These components are B- and T- lymphocytes. This kind of immunity is not immediate and develops slowly but it is long-lasting, since adaptive responses are able to rise more rapidly and intensely upon subsequent exposures to the pathogens. Adaptive immunity is constituted by very specific mechanisms of defense, which can be divided into active or passive. Active immunity results when exposure to a disease organism triggers the immune system to produce antibodies (Abs) against it. Exposure to the pathogenic agent can occurs through infection with the actual disease (resulting in natural immunity), or introduction of a killed or weakened form of the disease organism through vaccination (artificial immunity). Passive immunity is instead provided by Abs obtained from outside the body. It can be natural, when the Abs pass from mother to fetus via placenta or are acquired by infant from mother's milk. However, it can be also induced artificially through injection of Ab-containing blood products. The major advantage of passive immunity is that it develops and acts immediately but, on the other side, it usually lasts for a short period of few weeks or months. Despite this classification, innate and adaptive immune systems share many cellular and molecular components and are in constant contact, thus establishing a single highly integrated set of defenses against pathogens (*Baxter D, 2007*).

The practice of artificially injecting pathogens or parts of them in order to induce immunity against a pathogen or a foreign agent, is called vaccination and can be defined as one of the most important progress in the history of medicine. Indeed, vaccination is among humanity's greatest achievements for reducing death, disability, and inequity caused by infectious diseases. In the past century, the use of vaccines has improved the health and the life expectancy of millions of people, providing to eliminate smallpox, which is considered worldwide eradicated

since 1980, or at least diminish some of the worst scourges of the past as poliomyelitis, measles, pertussis, tetanus, yellow fever, and diphtheria. First attempts to prevent an infectious disease date back many centuries with the practice of variolation, used in many different countries to prevent smallpox. The first vaccine in its modern form was produced by Edward Jenner in 1796, when he officially provided to the scientific community evidences of the efficacy of a vaccine that produces Abs against smallpox. Thanks to his efforts, the principle of vaccination was extended and widely explored by scientists such as Pasteur and his successors. Vaccination effectively became a common practice in the late 19th and early 20th century, when the study of bacteriology prompted scientist to develop the earliest vaccines using whole organisms (e.g. pertussis and tuberculosis), or microbial toxins (e.g. tetanus and diphtheria toxins) to induce protective immunity (*André FE, 2003*).

Recently, the World Health Organization (WHO) has expanded the immunization program, increasing the coverage of vaccines and introducing new formulations, where the adjuvant portion has increased over time and now comprises about half of vaccines licensed or in clinical use. The immunization program includes the six childhood vaccines against diphtheria, tetanus, pertussis, measles, polio, and tuberculosis, plus more recently introduced vaccines against hepatitis B virus, *Haemophilus influenzae* type b, mumps, pneumococcal disease, rotavirus and rubella (*Global Vaccine Action Plan 2011-2020. World Health Organization, Geneva, 2013*). Moreover, regional, national, and high-risk occupational immunization programs have added vaccines for varicella (chickenpox), meningococcal infection, typhoid fever, herpes zoster, human papillomavirus, yellow fever and Japanese encephalitis (**Figure 1**). These new vaccines, which have become available in the last decade, were rolled out globally, helping raise life quality by averting post-infection disease such as blindness, deafness, and neurological disorders.

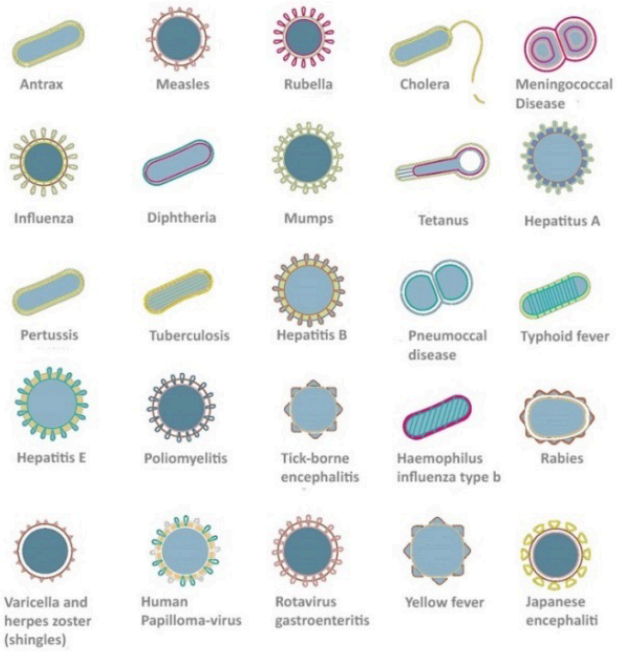

Figure 1. Vaccine-preventable infectious agents or diseases. There are now licensed vaccines being used to prevent, or contribute to the prevention and control of 25 vaccine-preventable infections (from *Global Vaccine Action Plan 2011-2020, World Health Organization, 2013*).

2. Vaccine immunology

The basal purpose of vaccination is to activate the immune system against specific pathogens or foreign agents, and build a specific immunological memory in order to protect the organism in case of future encounters with the cause of disease. After vaccination, the injected antigens (Ags) must provide sufficient danger signals to attract to the site of injection, cells of the innate immune system that trigger an inflammatory reaction, namely Ag presenting cells (APCs). APCs such as DCs, monocytes and neutrophils can recognise signals from the microenvironment through receptors termed as pattern recognition receptors (PRRs), whose engagement starts the immune cascade. PPRs are divided into five subclasses: a) TLRs, which are transmembrane proteins located at the cell surface or in endosomes; b) NOD-like receptors (NLRs), intracellular proteins that are primarily involved in antiviral responses; c) retinoic-inducible gene (RIG)-I-like receptors (RLRs), which are located intracellularly and are primarily involved in antiviral responses; d) C-type lectin receptors (CLRs), transmembrane receptors characterized by the presence of a carbohydrate-binding domain; e) absence in melanoma 2 (AIM2)-like receptors, involved in the detection of intracellular microbial DNA (**Figure 2**).

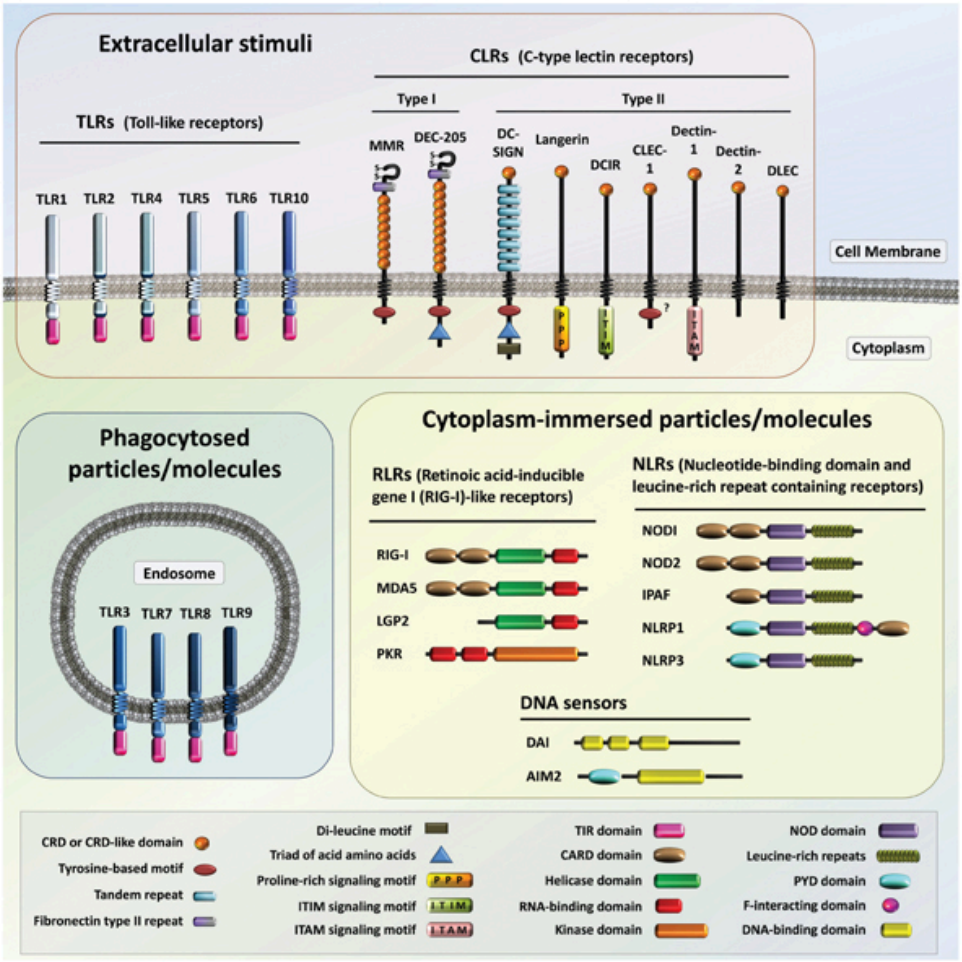

Figure 2. Schematic representation of the major classes of PRRs of the innate immune system (from *Miyaji EN et al., 2011*)**.**

PRRs recognize conserved structural moieties that are found in microorganisms, named PAMPs (Pathogen-Associated Molecular Patterns), and also non-infectious material that can cause tissue damage and endogenous molecules that are released during cellular injury or cell death, called DAMPs (Damage-Associated Molecular Patterns). While PAMPs are able to trigger classical inflammatory reactions against pathogens of viral or bacterial origin, DAMPs are involved in not-pathogen induced "sterile inflammation". In both cases, following ligands recognition, PRRs activate downstream signaling pathways that involve NF-κB, mitogen-activated protein kinase (MAPK), and type I interferon, resulting in the up-regulation of pro-inflammatory cytokines and chemokines (*Chen GY and Nuñez G, 2010*). This inflammatory microenvironment triggers the activation of APCs that migrate along the lymphatic vessels to the draining lymph nodes (dLNs), where they in turn activate T and B lymphocytes (*Plotkin S et al., 2012*).

One of the most important type of APCs are DCs. Since they play a crucial role in sensing foreign antigens and regulating the strength, quality, and persistence of the adaptive immune response,

they constitute the bridge between innate and adaptive immunity (*Pulendran B and Ahmed R, 2006; Tacken PJ et. al., 2007*). DCs capture and recognize Ags mostly through TLRs, whose binding to the Ag triggers DCs maturation. The maturation process occurs during DCs migration toward the dLNs and is characterized by Ag processing and modification of the expression of homing receptors on DCs surface (*Pardoll DM, 2002*). Upon recognition and internalization by DCs, exogenous Ags are degraded into small antigenic peptides in phagolysosomes and processed for presentation in the context of major histocompatibility complex (MHC)-II molecules. Differently, endogenous Ags are processed in proteasomes and presented in the context of MHC class I molecules. During this maturation process DCs increase the expression of MHC class I and II, co-stimulatory and adhesion molecules such as CD40, CD80 and CD86, becoming potent stimulators of T cells. In the dLNs, indeed, mature DCs present antigenic peptides to naive $CD4^+$ T cells in the context of MHC class II, or to cytotoxic $CD8^+$ T cells in the context of MHC class I. This process of T cell activation requires three different signals: the first is MHC-peptide and TCR interaction (signal 1), the second is given by the interactions between co-stimulatory and adhesion molecules such as CD80, CD86, CD40, CD54, CD58 on mature DCs (or other APCs) and CD28, CD154, CD2, CD11a on T cells (signal 2). Additional signals are provided by immunomodulatory cytokines (signal 3) produced by activated APCs that dictate the polarization of $CD4^+$ T cells towards T-helper 1 (Th1) or T-helper 2 (Th2) subtypes: interleukin-12 (IL-12) has an important role in the differentiation of Th1 cells, while IL-4 is crucial for Th2 differentiation. Many cytokines are produced by both Th subtypes, such as tumor necrosis factor α (TNFα), IL-3, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF). However, only Th1 effectors produce IL-2 and interferon-γ (IFN-γ), which principally mediate cellular immunity characterized by CTL activity, while Th2 cells produce IL-4, IL-5, IL-10, and IL-13, thus triggering strong humoral responses capable of eradicating extracellular parasites or cancer cells through Ab-dependent mechanisms, principally Ab-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Thus, the balance between Th1 and Th2 responses is a critical step to be considered during vaccine formulation in order to activate different arms of the immune system (*Beverley PC, 2002*). The most important effectors of a vaccine-induced immune response are essentially two: Abs, which constitute the humoral immune response and are capable of specific binding to toxins, pathogens and others extracellular Ags, and cytotoxic $CD8^+$ T lymphocytes, which belong to the cellular arm of immune response and limit the spread of diseases by recognizing and killing target cells directly or by secretion of specific cytokines (*Pardoll DM, 2002; Plotkin S et al., 2012*).

Once injected, vaccine Ags reach lymph node or spleen upon diffusion and/or in association to migrating DCs, and there activate B cells. Naive B cells generated in the bone marrow circulate through the body until they encounter a protein Ag recognized by their specific surface immunoglobulin M (IgM) receptor. Ag recognition triggers B cell activation and Ag-specific B cell migration towards the outer T cell zone of secondary lymphoid tissues, where they are exposed to recently activated DCs and T cells. These interactions provide signals, which rapidly drive B cell differentiation into plasma cells secreting low-affinity germline Abs (*MacLennan IC et al., 2003*) and Ig class-switch recombination from IgM toward IgG, IgA, or IgE, owing to $CD4^+$ Th1 and/or Th2 cells essential helper function (extrafollicular reaction). In this phase, the interaction between $CD4^+$ helper cells and B cells skew class-switch recombination into particular Ig classes and subclasses. In rodents, IFN-γ producing Th1 T cells promote switching towards IgG_{2a}, whereas Th2 cells support the generation of IgG_1 and IgE (via IL-4) and IgG_{2b} and IgG_3 (via TGF-β) (**Figure 3**) The extrafollicular reaction is rapid and short-lived, and leads to the production of Abs of germline affinity, in particular IgM, detectable at low levels in the serum. Following extrafollicular reaction, Ag-specific B cells are attracted by follicular dendritic cells (FDCs) in specialized areas, where they are in deep contact with Ag-specific follicular T helper (Tfh) cells and proliferate in structures called germinal center (GCs). The interaction between B cells, Tfh and FDCs in germinal center induces clonal proliferation of B cells, in association with Ig class-switch recombination from IgM towards IgG, IgA, or IgE, and maturation of the affinity for the specific Ag. This process results into a massive production of Abs of a higher Ag binding ability (*De Silva NS et al., 2015*). Since the development of the GC reaction generally requires a couple of weeks, hypermutated IgGs specific for protein Ags first appear in the blood 10-14 days after priming, with a peak value usually at 4 weeks after immunization. GC reaction terminates within maximum 6 weeks, a period during which a large number of Ag-specific plasma cells have been generated. Some of these plasma cells exit the lymph nodes/spleen and enter the blood stream secreting large amounts of specific Abs, others migrate to the bone marrow, where they survive as memory B cells (*McHeyzer-Williams M et al., 2011*). Those memory cells are reactivated in secondary immune responses, that consist in a rapid increase of IgG titer by secondary exposure to the Ag. The generation and the maintenance of these memory B cells repertoire is one of the major goal of vaccination (*Plotkin S et al., 2012*).

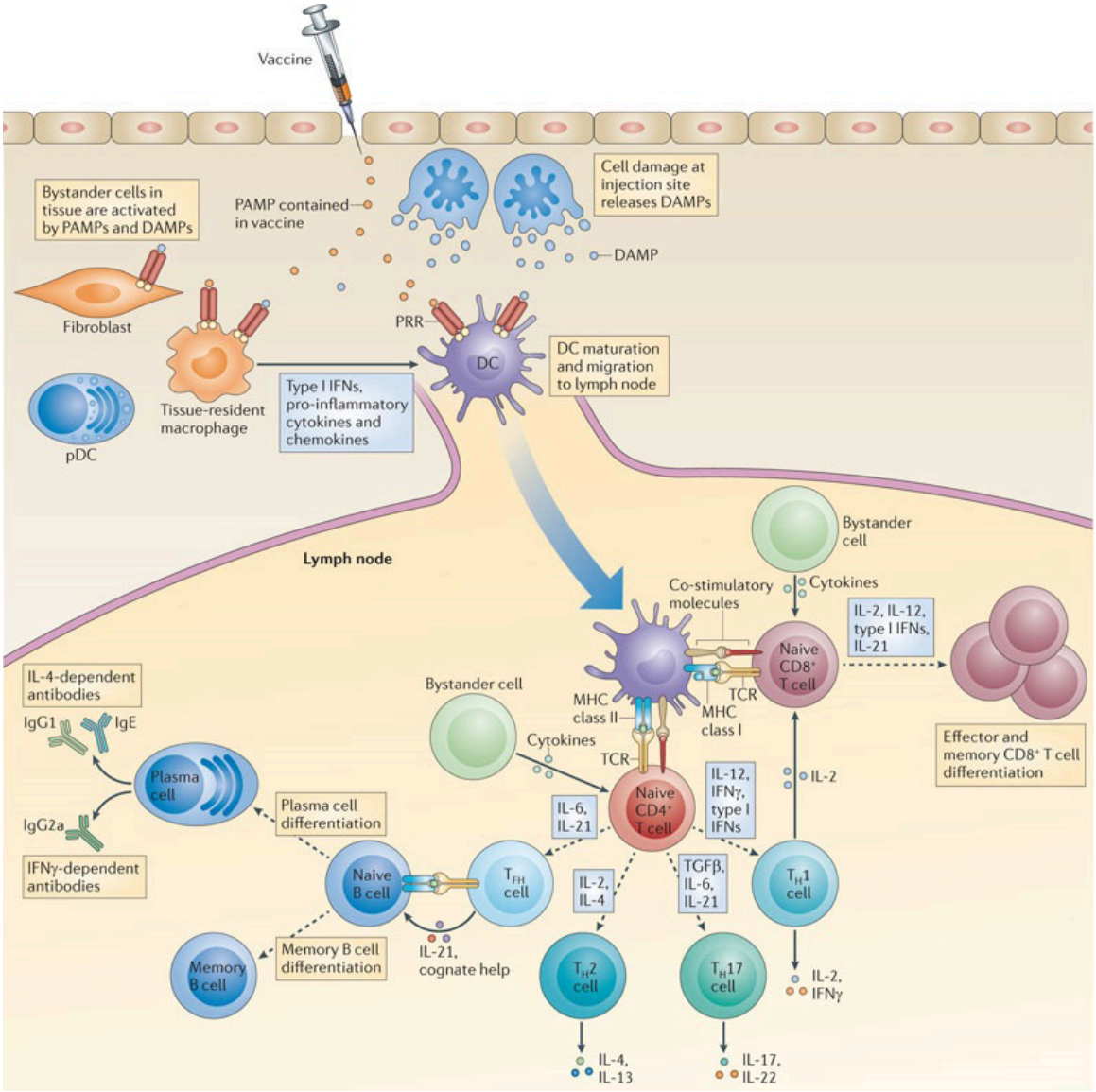

Figure 3. Immunological cascade following vaccine injection. Vaccines may contain PAMPs or may induce the local release of DAMPs, which are detected directly by PRRs expressed by DCs, leading to their activation, maturation and migration to the lymph nodes. Alternatively, PRR-mediated recognition of PAMPs and DAMPs by bystander cells may induce the release of cytokines, which cooperate in the activation and orientation of the DC response. In the lymph nodes, the activated DCs may present Ags to T cells, provide them with co-stimulatory signals and stimulate their differentiation by inducing a favorable cytokine milieu (from *Desmet CJ et al., 2012*).

3. Types of vaccines

Starting from the earliest vaccine against smallpox produced by Jenner in 1796, different types of vaccines have been developed and employed in the history of medicine. Jenner's smallpox vaccine was indeed the first example of attenuated vaccine, which contains a weakened form of the living virus that does not cause serious disease in people with healthy immune systems. The attenuation process consists in growing generations of viruses in foreign hosts, such as tissue cultures, embrionated eggs or animals in which they do not reproduce very well. In this way viruses evolve accumulating mutation in order to adapt to the new hostile environment, becoming weaker with respect to their natural host, the human being. Because live attenuated vaccines (e.g. adenovirus, Bacillus Calmette–Guerin, mumps, rubella, rotavirus, *Salmonella typhi* Ty21a, varicella, and yellow fever) are very close to the natural infectious agents, they are very efficient in activating the immune system eliciting strong cellular and Ab responses, and often confer life-long immunity with only one or two doses. Another category of vaccines are inactivated vaccines, which are obtained by killing the disease-causing microbes with chemicals, heat, or radiations. The inactivated polio vaccine is an example of this type of vaccines, which often require multiple doses to build up and/or maintain immunity. Of note, such vaccines are more stable and safer than live vaccines since the dead microbes can not mutate back to their disease-causing state. Toxoid vaccines are another category of vaccines, efficient for immunization against toxin-based diseases. Toxoids are "detoxified" toxins, weakened by treating them with formalin, that render them inactive and thus safe for use in vaccines. When the immune system receives a vaccine containing a toxoid, it learns how to fight off the natural toxin, without causing illness. Toxoid-vaccine are still commonly used, the DTaP vaccine, for example, containing diphtheria and tetanus toxoids combined with pertussis Ags (*Baxter D, 2007*).

Since the previously described categories of vaccines still contain traces of the pathogens, they maintain, although extremely low, a certain level of risk of side effects for humans. The imperative of vaccine safety has led to the abandon of development of whole-cell inactivated or live attenuated vaccines and to increase efforts to develop subunit or non-replicating recombinant vaccines. Moreover, the "genomic revolution" opens new opportunities and knowledge for the rational design of novel vaccines. With the development of recombinant DNA technology, genomics and proteomics and the improvement in the field of immunology, some purified components of the pathogens have been found to be immunogenic and thus suitable for efficient immunization. Those discoveries led to the development of the so-called subunit vaccines, which include only parts of a virus or bacteria that best stimulate the immune system,

instead of the entire germ. Because these vaccines contain only the essential Ags, side effects are less common. Subunit vaccines created via genetic engineering are called recombinant vaccines and are constituted by Ags expressed in a heterologous expression system (*E. coli*, yeast, insect etc.) using recombinant protein expression technologies. One of the best examples of recombinant protein vaccine currently in use in humans is the vaccine against Hepatitis B Virus (HBV). Genes coding for the hepatitis B surface Ag (HBsAg) were inserted into the yeast *Saccharomyces cerevisiae*, and the produced Ags are collected and purified for use in the vaccine. DNA vaccines are another sub-group of subunit vaccines, consisting of DNA coding for a particular Ags, which are directly injected into the muscle. The DNA itself inserts into the individual's cells, which then produce the Ag from the infectious agent. Since this Ag is foreign, it generates an immune response. This type of vaccine has the benefit of being safe since it does not contain the microbe but just copies of a few of its genes, and are relatively easy and inexpensive to design and produce, since DNA is very stable and easy to manufacture, but is still experimental because no DNA-based vaccines have been shown to elicit the substantial immune response required to prevent infection. Conjugate vaccines are subunit vaccines developed to fight bacteria that have an outer coating of polysaccharides that disguises the Ag, making it hard for immune system to recognize and respond. Conjugate vaccines are designed to be effective against these types of bacteria because they are based on conjugation of the polysaccharides to Ags against which the immune system responds very well *(Rappuoli R et al. 2011, Rappuoli R et al. 2012)* (**Table 1**). In general, subunit vaccines composed by purified, synthetic and recombinant Ags are deprived of the endogenous danger signals, and are therefore less immunogenic than naturally adjuvanted whole-cell or live vaccines. Attempts over the years to improve the quality and the purity of vaccines, in order to improve safety and reduce their adverse reactivity, have paradoxically resulted in inadequate vaccine immunogenicity. As consequence, modern subunit vaccines require the addiction of components to potentiate the immune response: such components are known as adjuvants.

Vaccine type	Description	Advantages	Drawbacks	Examples
Killed microorganism	The causative agent is inactivated by chemical or physical treatments	Efficacious	Some pathogens are difficult or almost impossible to cultivate in a scalable setting; regulatory authorities require high safety and quality standards for all new vaccine formulations, so obtaining approval is difficult	Polio vaccine (e.g. developed by Jonas Salk); influenza vaccine; oral cholera vaccine
Live attenuated microorganism	The causative agent is live, but it has lost the ability to cause disease	Efficacious; can induce protective immune response	Some pathogens are difficult or almost impossible to cultivate in a scalable setting; regulatory authorities require high safety and quality standards for all new vaccine formulations, so obtaining approval is difficult	Polio vaccine (developed by Albert Sabin); intranasal influenza vaccine (cold adapted); MMRV vaccine
Subunit	Vaccines contain purified portions of the causative agents	Toxins are inactivated chemically. If not properly inactivated they can cause disease; such inactivated vaccines cannot provoke the disease; if recombinant form of the selected components are used, the pathogens need not to be cultivated	Identification of the few protective components from the pool of molecules in the pathogen is usually complex and time consuming	Diphteria, tetanus and pertossius toxoids; hepatitis B vaccine; acellular pertossius vaccine
Subunit conjugated	A polysaccharide component of the causative agent is chemically linked to a protein carrier	The conjugated polysaccharide, which is poorly immunogenic on its own, becomes immunogenic	Need to grow the pathogen *in vitro* to obtain the capsular polysaccharide; capsule not always immunogenic; too many capsule types	Hib vaccine; PnC vaccine; MenACWY vaccine

Table 1. Different types of vaccines. Abbreviations: MMRV= measles, mumps, rubella, and varicella combination, Hib= *Haemophilus influenza* type b. PnC= pneumococcal conjugate. MenACWY= meningococcal conjugate for group A, C, W-135, and Y (Modified from *Rappuoli R et al. 2011)*.

4. Vaccine adjuvants

An adjuvant is a compound which is added to the vaccine formulation in order to increase its efficacy by modulating, enhancing, or extending the immune response. The word "adjuvant" comes from the latin *adjuvare* and means "to help." Adjuvants help activating the immune system, allowing the Ags present in vaccine formulations to induce long-term protective immunity. The emerging popularity of poorly immunogenic subunit vaccines has underlined the importance of discovering new and potent adjuvants, which are fundamental to improve vaccine efficacy. Their main function in vaccine formulations is the enhancement of Ag immunogenicity, allowing dose sparing and the reduction of numbers of booster immunizations required to achieve and modulate the desired immune responses. The addition of certain kind of adjuvants to vaccines permits to evoke both humoral and cellular immune responses, improving vaccines efficacy in poor responder populations including newborns, immunocompromised individuals, and elderly (*Petrovsky N and Aguilar JC, 2004*). Moreover, the addition of certain adjuvants in vaccine formulation has been demonstrated to accelerate the development of immune responses and to increase the duration of protection. Indeed, the portion of vaccines formulated with added adjuvant has increased over time and now comprises about half of vaccines either licensed or under clinical testing.

The history of adjuvant discovery begins in 1893, when the oncologist William Coley showed that the administration of a mixture of killed bacteria (Coley's toxins) could in some cases cure certain forms of cancer. Two decades later, in 1925, the French veterinarian Gaston Ramon noticed that the administration of diphtheria toxoid to horses, in combination with a variety of substances, including starch, plant extracts, or fish oils, increased vaccine efficacy, producing a more robust immune response than that obtained with the Ag alone (*Ramon G, 1925*). The key milestone in adjuvant history was the discovery, in 1926, by Alexander Glenny and coworkers of the potent adjuvant effect of aluminum potassium sulfate, or Alum (*Glenny A et al., 1926*). This date marked the beginning of the supremacy of Alum, which has been using as an adjuvant in a wide number of human vaccines, in the forms of aluminum oxyhydroxide or hydroxyphosphase. About the same period, in 1930, Jules Freund developed a powerful adjuvant composed of a water-in-mineral oil emulsion containing heat-killed mycobacteria (*Mycobacterium tuberculosis* or others) *(Opie EL, 1937).* Although highly effective, Complete Freund's Adjuvant (CFA) had never become able to compete with Alum as adjuvant in human vaccines since it is very reactogenic and frequently induces granulomas, sterile abscesses, and ulcerative necrosis at the site of injection.

All these early empirical observations contribute to underline the key role and the potentiality of adjuvants in strengthening and enhancing the immune system. Nevertheless, the inadequate knowledge of basic immunology, associated to a scarce research focused on adjuvant mechanism of action has led to a slowdown in adjuvant development and to a steady state phase of complete leadership of Alum, which was surprisingly employed for over 80 years without knowing its mechanism of action (**Figure 4**).

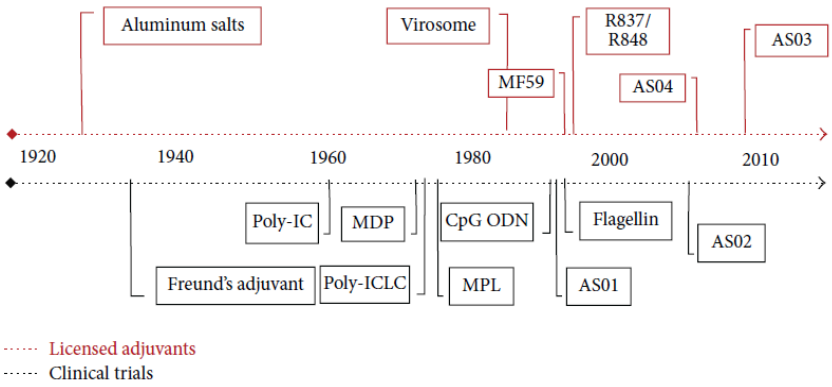

Figure 4. Timeline of vaccine adjuvant discovery (from *Apostólico Jde S, 2016*)**.**

4.1 Mechanisms of action of adjuvants

A major limitation to advance in vaccine technology following Alum discovery and diffusion, was the incomplete understanding of the cellular and molecular mechanisms by which adjuvants function. Profiling the mechanism of action of newly discovered adjuvants is a critical point to design new efficient vaccination strategies. In recent years, several studies have been slowly revealing the secrets of adjuvant activity and considerable advances have been made in understanding the mechanisms of action of various adjuvants, particularly the activation of innate immunity via various mechanisms. It appears that adjuvants activate innate immune responses to create a local immunocompetent environment at the injection site. Depending on the type of innate responses activated, adjuvants can model the quality and quantity of adaptive immune responses. The main mechanisms that have been described so far to explain adjuvants action, are the following:

a) Sustained release of Ag at the site of injection (depot effect).

One of the first recognized mechanisms of action of vaccine adjuvants is the formation of a depot, and the consequent Ag trapping at the injection site. The prolonged deposition and slow release of Ag from the injection site ensure a constant local stimulation of inflammatory cells of the innate immune system. This so called depot effect, is due to the chemical composition of the

adjuvant itself and is a characteristic feature of emulsions, such as the water-in-oil emulsion (e.g. CFA) and biodegradable micro- and nano-particles, such as liposomes (e.g. CAF01, *Henriksen-Lacey M. et al., 2010*)

b) Cellular recruitment at the site of injection and up-regulation of cytokines and chemokines.

A consequence of the prolonged retention of the Ag at the site of injection is the recruitment of various inflammatory cells. These recruited cells secrete cytokines and chemokines, which in turn attract other immune cells, causing the formation of a local immunocompetent environment at the injection site. Moreover, chemokines, which play a critical role in tissue-specific migration of immune cells, were shown to be up-regulated by adjuvants at the injection site. For example, MF59 significantly up-regulates the expression of C-C chemokine receptor type 2 (CCR2), a receptor for chemokine (C-C motif) ligand 2 (CCL2), which is involved in the monocyte infiltration process (*Seubert A et al., 2008*). MF59-mediated immune cell recruitment to the injection site has been studied in detail showing the enrollment of neutrophils, monocytes, eosinophils, macrophages followed by DCs after intramuscular injection in mice. The recruited cells, especially neutrophils, monocytes, and B cells, take up both Ag and adjuvant and traffic to dLNs (*Calabro S et al., 2011*).

c) Increased Ag uptake and presentation by APC.

Efficient Ag capture, processing and presentation by APCs is important for the stimulation of adaptive immune response. It was held that many adjuvants act by "targeting" Ags to APCs resulting in enhanced Ag presentation by MHC (*Schijns VE and Lavelle EC, 2011*). In 1985, Alum was shown to increase Ag uptake by DCs and alter the magnitude and duration of Ag presentation (*Mannhalter JW et al., 1985; Morefield GL et al., 2005)*, but only recently *Flach et al. (2011)* have shown that Alum does not enter DCs directly but rather delivers the Ag via abortive phagocytosis, interacting with membrane lipids on DCs, leading to lipid sorting and Ag uptake *(Flach TL et al., 2011*). Although Ag size seems to play an important role in modulating the Ag presentation efficiency, the role of adjuvant-induced increased Ag presentation has not been clearly evaluated yet, and the knowledge regarding the role of this adjuvant mechanism is still limited.

d) Activation and maturation of APCs.

APCs and in particular DCs play a fundamental role in initiating and controlling the quality and strength of the immune response, and thus represent the ideal target for adjuvants. One of the most wanted effects of a vaccine is indeed to promote APC activation; in particular, targeting DCs induces their maturation and enhances MHC class II and co-stimulatory molecules expression. Adjuvants activate DCs either through direct interactions or through cellular

intermediates such as monocytes, macrophages, or granulocytes that induce cytokines to generate a local immunostimulatory environment. As already described, Ag recognition through PRRs, and in particular TLRs, triggers DCs maturation and consequently most studies are focusing on improving vaccine formulations by the addition of TLR ligands as adjuvants. One example is AS04, which has been shown to interact with TLR4, inducing maturation of DCs and stimulating their migration to the dLNs (*Didierlaurent AM et al., 2009*).

e) Ag delivery to the draining lymph nodes.

Lymph nodes are peripheral lymphoid organs where activated DCs interact with T and B cells to initiate the adaptive immune response. Drainage of Ags to LNs following injection depends on different factors, such as injection route and particles size. It has been shown that the arrival of Ag in free form or in association with APCs has crucial effects on the targeting of different cell populations. Differently from particles larger than 200-500 nm, which do not efficiently enter lymphatic capillaries in free form but must be carried by migratory DCs subsets, small-size nanoparticles are able to freely enter lymphatic capillaries. Some adjuvants were proposed to act by driving the Ags to LNs, improving their chance to evoke effective immune responses. In particular, this mechanism was proposed for nanoparticles, such as Virus-like particles (VLPs), which have the property to drain freely and deliver Ags to LNs due to their small size (200 nm) (**Figure 5**). Once in the LNs, these small molecules have the possibility to target directly LN-resident DCs, such as $CD8^+$ DCs, which have been implicated as key APC for priming $CD8^+$ T cells (*Manlova V et al., 2008*). Of particular interest is the possibility of associating the Ag with stimulatory molecules (e.g. TLRs agonist); this co-delivery allows a more focused activation of target cells of the innate immune system (*Bachmann MF et al., 2010*).

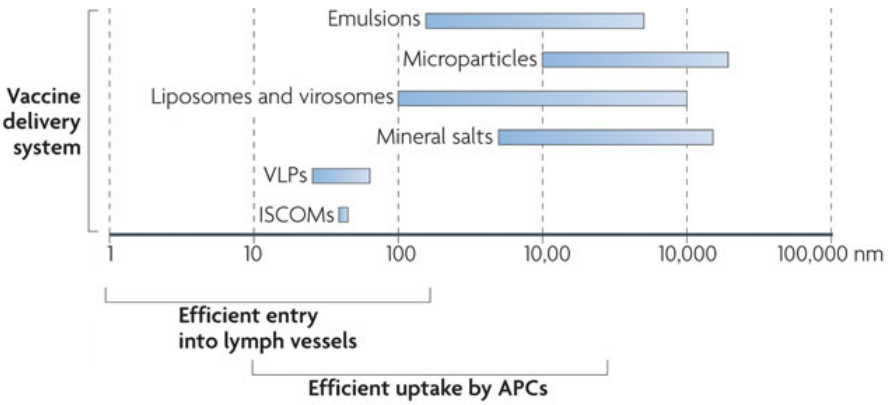

Figure 5. The sizes of adjuvant delivery systems (modified from *Bachmann MF et al., 2010)*.

f) Activation of inflammasomes

This last mechanism of action has been proposed and investigated only recently. Inflammasomes are multiprotein complexes that contain a PRR, typically a member of the NLR family, which after sensing its agonist oligomerizes and recruits the apoptosis-related speck-like protein containing a caspase activation and recruitment domain (CARD; ASC protein). The immediate effect of responses involving the inflammasome is to activate the inflammatory process by promoting the secretion of the proinflammatory cytokines. There are currently four characterized inflammasomes, named basing on the PRRs involved (*Schroderand K and Tschopp J, 2010*). Among them, the pyrin-domain-containing 3 (NLRP3) is the most studied inflammasome receptor in regards to adjuvant mechanisms. The NLRP3 inflammasome has been demonstrated to sense various stimuli including DAMPs, environmental irritants and metabolic stress. In particular, it has been shown to play a critical role in the adjuvant activity of Alum *in vitro*. Several lines of evidence confirm that, at the site of Alum injection, a catabolic product of host DNA, uric acid, and ATP released after cell damage or necrosis act as danger signals for activation of NLRP3. Activation of NLRP3 inflammasome induces caspase-1 activation, which in turn cleaves precursor of IL-1β, IL-18 and IL-33 to their active forms, which are potent stimulators of the adaptive immunity. Contrary to *in vitro* studies, the role of inflammasomes in the adjuvant activity of Alum *in vivo* has yielded conflicting results (*Bergmann-Leitner ES and Leitner WW, 2014*), which can be likely attributed to the differences in the nature of Alum used in different studies, immunization protocols, and the mouse strains used (*De Gregorio E et al., 2008; Marrack P et al., 2009*).

Taken together, a detailed knowledge of the mechanisms of action of adjuvants is very important in the rational design of vaccines. A deeper understanding of these mechanisms of action will provide important information also about their safety, which is a major concern when it comes to adjuvant approval for human use. Currently, a large number of experimental adjuvants are undergoing preclinical or clinical stages of development, although their mechanisms of action are not fully characterized, but recent progresses in the field of immunology will allow to define and clarify many of these aspects (**Figure 6**).

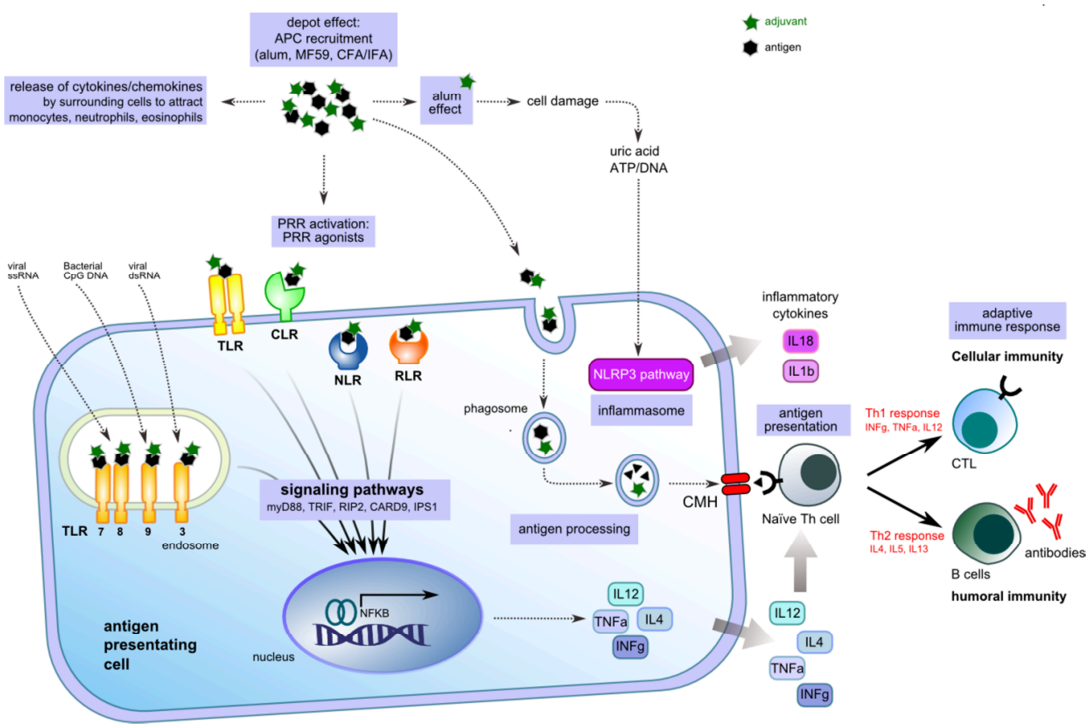

Figure 6. Proposed mechanisms of action of vaccine adjuvants. Adjuvants can mediate their activity through different mechanisms of action: by forming a depot at the site of injection, creating a proinflammatory microenvironment with transient production of cytokines and chemokines, leading to recruitment of cells of the innate immune compartment. By activating PRRs both on the surface and intracellularly on APCs, specially DCs, which are influenced by adjuvants to upregulate the production of some cytokines and chemokines, with a concomitant downregulation of others, globally influencing the balance between Th1 and Th2 responses. Finally, adjuvants can also work by activating inflammasome, leading to the production of proinflammatory cytokines IL-1β and IL-18.

4.2 The ideal vaccine adjuvant

Adjuvants improve vaccine effectiveness by modulating the immunogenicity of Ag delivered, and facilitating transport and presentation. In the process of developing and selecting new adjuvants, one of prime factor to assess is biocompatibility, which is the ability of a material to accomplish its appropriate action by producing the most suitable cellular or tissue response, without inducing any undesirable local or systemic effects. Most of the inflammatory reactions that follow vaccine injection are due to the adjuvant action, but they usually occur locally and transiently. Excessive adjuvant-induced inflammation is unsuitable and can cause both local reactogenicity, which manifests as pain, swelling, redness, ulceration and sterile abscess, and also systemic reactogenicity, which is characterized by lymphadenopathy, splenomegaly, nausea and vomiting, fever, myalgia, fatigue, headaches and can even culminate in allergic reactions and autoimmunity. Therefore, the main challenge of new adjuvants is to trigger sufficient local activation signals to increase humoral response, while avoiding both an excess of inflammatory

reactions and non-specific activation of immune cells. Immunogenicity is the fundamental feature that a novel adjuvant requires, and consists in promoting the crosstalk between the two arms of the immune response, both increasing the total functional Ab titer and eliciting cell-mediated immunity (CMI), thus stimulating a balanced response for Th1, Th2, Th17. In particular, an ideal adjuvant should aim at improving the effective engagement of T helper cells for optimizing the durability of Ab responses, but also at inducing effector $CD8^+$ cells. Cytotoxic responses have been demonstrated to be fundamental to kill intracellular pathogens such as malaria and human Immunodeficiency Virus (HIV), and to fight chronic infections (for example, from hepatitis C virus (HCV), tuberculosis and herpes simplex virus (HSV)) and cancer *(Reed SG et al., 2013)*. Data about cancer vaccination in preclinical and clinical studies firmly support the idea that protective and therapeutic immune responses are mainly based on Th1 cytokines, in particular IFN-γ, with the induction of a potent cytotoxic response. So far, most of the commonly used adjuvants in vaccines approved for human use, including Alum and oil-in-water-based emulsions, have failed in eliciting appropriate cytotoxic T-cell responses, and therefore this goal has become a key milestone for novel adjuvant development. Nonetheless, an ideal adjuvant should confer long-term protection, decreasing the dose of Ag and the total number of doses needed to achieve complete immunization. Induction of the right type of immune response for each disease is thus crucial for effective vaccination. Since adjuvants have a key role in shaping the immune response, regulating the balance between Th1 and Th2 responses and affecting the outcome of the immune response, their use in different vaccines should be optimized based on the wanted effect on the immune response. Taken together, an ideal adjuvant should be safe and efficient from a biological point of view, but it must also satisfy some physicochemical criteria. Stability is a main property that must be considered in the process of developing new adjuvants. These molecules must indeed be stable before administration under a broad spectrum of storage time/temperature/pH conditions in order to be efficiently employed in vaccine formulations. Moreover, a putative adjuvant must be also chemically and physically defined to ensure reproducible manufacturing and activity, and its production and formulation must be easy and cost- effective (*Reed SG et al., 2009*) (**Figure 7**).

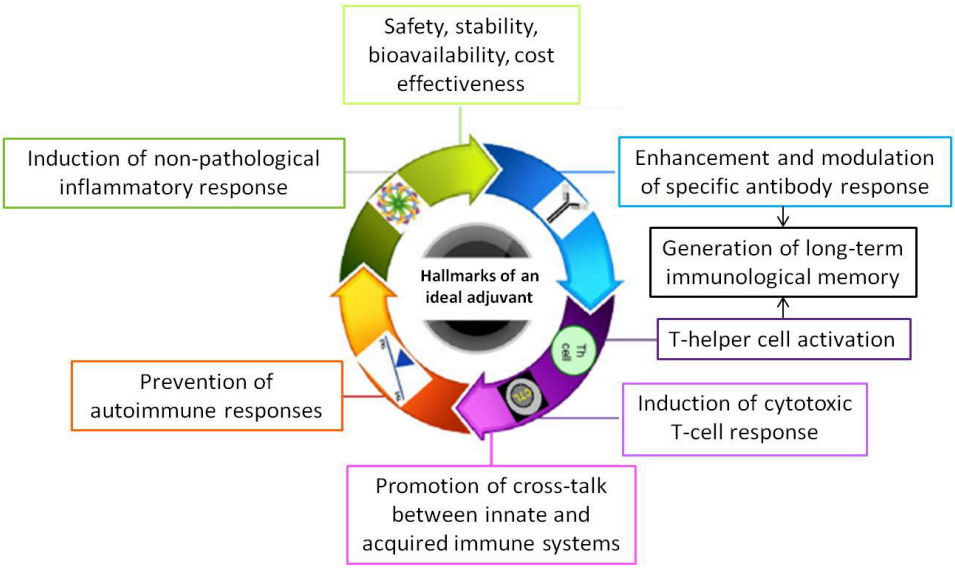

Figure 7. Schematic diagram showing the most important features of an ideal vaccine adjuvant (modified from *Chowdhury RR and Ghosh SK, 2012*).

4.3 Vaccine adjuvants classes

A wide pool of molecules with many different properties have been proposed and studied in the last 80 years as vaccine adjuvants. A variety of novel compounds with adjuvant properties are currently under evaluation and used in experimental settings, but only few of them are approved for clinical usage in human vaccines. The classification of such amount of molecules results sometimes hard, and different criteria may be used to group adjuvants in order to allow their rational comparison. Indeed adjuvants can be classified according to their physicochemical properties, origin or mechanisms of action. The most commonly reported classification of adjuvants relies on their dominant mechanism of action and divides them into two main groups: delivery systems and immune potentiators (immunostimulatory adjuvants) (*O'Hagan DT and Singh M, 2003*). Delivery systems act mainly by providing an Ag depot and helping to co-localize Ags with APCs, while immuno-stimulatory adjuvants have the intrinsic ability to activate cells of the innate immune system (*Pashine A et al., 2005*). However, more complex formulations are increasingly being developed in which delivery systems are exploited both for the delivery of Ags and also for the delivery of co-administered immunostimulatory adjuvants and immune potentiators, rendering this classification hard to apply. In this thesis, the main classes of adjuvants will be described and conventionally classified into the following categories: mineral compounds, emulsions and surfactants-based formulations, ISCOMs, liposomes, TLR agonists and natural polymers (**Figure 8**).

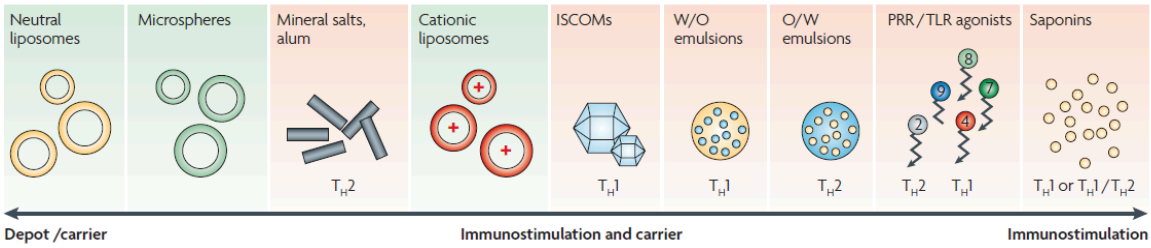

Figure 8. Properties of different classes of adjuvants. The main classes of adjuvants with respect to their depot/carrier and immunostimulatory properties are shown. Some compounds represented with red background can have immunostimulatory properties beyond their ability to trigger global immune stimulation, by directing responses specifically towards a Th1 or Th2 response. (from *Guy B et al., 2007*).

4.3.1 Mineral salts: Alum

Aluminum salts are mineral compounds consisting of crystalline nano-particles that aggregate to form a heterogeneous dispersion. Usually defined under the name of Alum, aluminum salts adjuvants are gels based on aluminium oxyhydroxide (aluminium hydroxide gel), aluminium hydroxyphosphate (aluminium phosphate gel), or various proprietary salts such as aluminium hydroxyl-sulfate. Despite widely employed in billion doses of vaccines since 1920, the mechanism of action of Alum has only begun to be elucidated and is, as previously mentioned, still discussed. The basic effect of Alum is the absorption of the Ag, due to electrostatic and hydrophobic attraction, that results in a delayed clearing of the Ag from the injection site. Knowledge about Alum adjuvant's action progressed substantially in recent decades, suggesting that its adjuvant effect could result from several different mechanisms *(Wen Y and Shi Y, 2016)*. Alum has been indeed demonstrated to enhance Ag uptake by DCs and to promote cell recruitment at the injection site, but it has also been shown to act as a stress-inducing agent producing necrosis and uric acid, a danger signal that activates the NLRP3 inflammasome (*Lambrecht BN et al., 2009*). Another recently proposed mechanism describes that Alum salts induce lipid reordering on the cell membrane of DCs, improving their Ag uptake capability (*Flach TL et al., 2011*). How much these mechanisms are of relevance to the total effect of Alum's adjuvanticity requires further analyses. Nonetheless, Alum-based adjuvants, alone or in combination with additional immune activators, remain the most widely employed adjuvants for several licensed vaccines, including those against diphtheria-pertussis-tetanus, diphtheria-tetanus (DT), DT combined with HBV, *Haemophilus influenzae B* or inactivated polio virus, Hepatitis A, *Streptococcus pneumoniae*, *Neisseria meningitidis* and human papilloma virus (HPV) (*Reed SG et al., 2009; Mbow ML et al., 2010*). Despite its low cost, stability, safety and strong stimulatory effects on the immune response, the mayor disadvantage of this very effective adjuvant is that it mostly contributes to the induction of a strong Th2 immune response, but has

little capacity to induce cell-mediated Th1 or cytotoxic $CD8^+$ T-cell responses, which limits its application in vaccines against intracellular pathogens. Indeed, Alum is not ideal for small peptide vaccines or for use with recombinant proteins. Moreover, although very stable and easy to store, vaccines containing this adjuvant cannot be lyophilized or frozen, because these procedures lead to loss of potency and thus limit vaccines shelf life and storage conditions. Finally, even though Alum is regarded as safe, it is able to induce severe local side effects and tissue reactions such as sterile abscesses, subcutaneous nodules, granulomas, and has been thought to induce hypersensitivity and myofasciitis.

4.3.2 Emulsions: CFA/IFA, Montanide and Addavax

An emulsion is a mixture of two immiscible phases: the dispersed phase and the continuous phase, which are stabilized by one or more surfactant(s). Emulsion adjuvants can be divided in water-in-oil emulsions (W/O) and oil-in-water emulsions (O/W), depending on the volume fractions of each phase and on the type of surfactant used. The first class of emulsions consists of nano-sized water droplets dispersed in an oil phase in a detergent-stabilized emulsion. One example of W/O emulsion is Freund´s Adjuvant, a solution of mineral oil emulsified in water with a manide mono-oleate surfactant. Freund´s Adjuvant can be named as complete or incomplete (IFA), depending on the presence or absence of inactivated and dried mycobacteria (usually *Mycobacterium tuberculosis*) in its formulation. This adjuvant was firstly described over 80 years ago and is known to elicit strong immune responses due to the attraction of macrophages and other cells to the injection site by mycobacteria contained in CFA. Injection of Ag in CFA induces a Th1-dominated response when compared to injection in IFA, which induces a Th2-dominated response. The main disadvantage of Freund's Adjuvant is that it can cause granulomas, inflammation and lesions at the inoculation site. To minimize side effects, CFA is used for the initial injections, while IFA is the adjuvant of choice for subsequent immunizations. CFA/IFA-associated reactogenicity renders this adjuvant not acceptable for licensure and clinical usage, but thus far it remains the gold standard for all adjuvant potency comparisons performed in animals. A new generation of W/O emulsions is represented by Montanide ISA 51 and Montanide ISA 720 *(Aucouturier J et al., 2002*). Montanide ISA 51 is a mixture (50:50 O:W ratio) of a mineral oil with a mannide mono-oleate family surfactant, and is mainly used in cancer vaccine application since it has been shown to induce a Th1-biased response. On the other hand, Montanide ISA 720 is a mix of vegetable oil with the same type of surfactant, which also gives W/O emulsions (70:30 O:W ratio) and is mainly used in infectious disease vaccine application. These emulsions are currently under testing in various phase I, II or III clinical trials all over the world in therapeutic vaccines mainly against malaria, cancer or acquired immunodeficiency

syndrome, and have been observed to generate mild to moderate local adverse events. Montanide has thus a generally high safety profile suitable for human injection, since adverse events are generally mild and transient such as redness, granulomas or pain at injection site. A non-small-cell lung cancer (NSCLC) vaccine containing Montanide ISA 51 as adjuvant (CimaVax EGF, Bioven) was recently registered in Cuba, Chile, Colombia, Perù, and Paraguay (*Crombet Ramos T et al., 2015*). One example of O/W emulsion is Addavax, a squalene-based adjuvant consisting of nano-emulsification of two components: Sorbitan trioleate (0.5% w/v) in squalene oil (5% v/v) and Tween 80 (0.5% w/v) in sodium citrate buffer (10 mM, pH 6.5). This formulation is an analog of MF59, a registered trademark of Novartis (Wilson-Welder JH *et al*., 2009), one of the most promising candidates to successfully replace Alum in future and already approved for human use in Europe (*Mbow ML et al., 2010*). Addavax, similarly to MF59, generates higher Ab titers with more balanced IgG_1:IgG_{2a} responses than those obtained with Alum. The mechanisms of MF59 adjuvanticity seems to be in part due to a direct delivery of immunogens to APCs *in vivo*. However, it has been demonstrated that Ag biodistribution and clearance from the site of injection are not influenced by MF59, suggesting that the adjuvant does not induce any Ag depot (*Dupuis M et al., 1999*). MF59 is not able to directly activate DCs, but its intramuscular administration triggers infiltration and activation of mature macrophages, which engulf and transport the Ag to the dLNs, thus resulting in a more efficient T cell priming (*Dupuis M et al., 2001*). Additionally, MF59 triggers a local immunostimulatory microenvironment characterized by the expression of several cytokines, which may enhance the recruitment of granulocytes and accelerate the differentiation of monocytes towards DCs, resulting in their migration into the dLNs. (*Tritto E et al., 2009*). Since Addavax and MF59 contain squalene, which derives from biodegradable plant oil and is thus easily metabolized, they both show an overall acceptable safety profile; however, similarly to most emulsion adjuvants, they also have the problem of local reactogenicity at the injection site. AS03 (Adjuvant System 03) is another squalene-based O/W emulsions composed of α-tocopherol and polysorbate 80. AS03 has been shown to enhance the vaccine-induced Ag-specific adaptive response by activating the innate immune system locally and by increasing Ag uptake and presentation in dLNs, a process that is modulated by the presence of α-tocopherol (*Garçon N, et al., 2012*). Moreover, α-tocopherol is important for promoting the expression and modulation of cytokines and chemokines, Ag loading in monocytes and increased recruitment of granulocytes in the dLNs (*Morel S et al., 2011*). Together with MF59, AS03 have been approved as adjuvant for influenza vaccines in Europe and Canada (Fluad®, Novartis and Pandemrix, GlaxoSmithKline Biologicals) since both have shown to

allow consistent Ag sparing, an important factor particularly during pandemics (*Levitz SM and Golenbock DT, 2012*).

4.3.3 Saponin-based adjuvants: Quil-A, ISCOMs and ISCOMATRIX

A range of plant-derived compounds, known as saponins, have been identified to have immunological actions. Saponins from *Rhamnaceae*, *Araliaceae*, *Polygalaceae* and *Fabaceae* plant families have all been reported to have adjuvant activity. An extensively characterized plant saponin from an adjuvant perspective is QS-21. QS-21 is derived from Quil-A, a mixture of triterpenoid glycosides derived from the bark of the South American soap bark tree, *Quillaja saponaria.* It has been hypothesized that the adjuvant effect of saponins is related to pore formation, which may allow Ags to gain access to the endogenous pathway of Ag presentation, promoting a CTL response (Sjölander *S et al., 2001*). The exact mechanism of action is still unknown, but QS-21 has been shown to induce inflammatory cytokines, IgG_{2a} Abs and $CD8^+$ T cells in mice, consistent with a Th1-biased response (*Kensil CR et al. 1995*); these features potentially make saponins ideal for use in subunit vaccines and vaccines directed against intracellular pathogens, as well as for therapeutic cancer vaccines. Numerous clinical trials have been conducted using *Quillaja saponaria* as a vaccine adjuvant, but they showed serious drawbacks such as high toxicity, undesirable hemolytic effect due to its potent ability to lyse cell membranes and instability in aqueous phase, which limits its use as adjuvant in human vaccination (*Sun HX et al, 2009*). The immunostimulatory fractions from Quil-A have been combined with cholesterol and phospholipids to form ISCOMs (Sjölander A *et al., 1998*). The principal advantage of the preparation of ISCOMS is to allow a reduction in the dose of the haemolytic Quil-A adjuvant, and to target the formulation directly to APC. The classical ISCOM technology required incorporation of vaccine Ags into the structure, which not only restricted the types of Ags that could be used, but was also a laborious process difficult to control. For these reasons, most current applications employ a mixture of soluble Ags and the Ag-free particle, such as ISCOMATRIX (*Coffman RL et al., 2010*). ISCOMs and ISCOMATRIX combine Ag presentation by both MHC class I and class II pathways, and immunostimulatory features of the saponin and have been demonstrated to induce both Th1 and Th2 responses that result in robust and effective Ab and $CD8^+$ T cell responses. Although there are currently registered ISCOMs vaccines for veterinary applications, adjuvant properties and safety of ISCOMs and ISCOMATRIX are currently being evaluated in clinical trials of influenza, HCV, HPV, and further investigation are needed before their application in human vaccines (*Lövgren BK et al., 2011*).

4.3.4 Liposomes

Liposome-like particles are sphere-shaped vesicles of varying size consisting primarily of amphipathic phospholipids. Due to the chemical properties of phospholipids, they automatically form a spherical lipid bilayer with an aqueous inner compartment that can be loaded with Ags. Liposomes were first described as vaccine adjuvants in 1974, when it was shown that immunization of mice with DT adjuvanted with phospholipid-based liposomes resulted in increased Ab titers as compared to immunization with non-adjuvanted DT (*Allison AG et al., 1974*). Following these studies, the use of liposomes as vaccine adjuvants has been intensively investigated and clinically tested. The adjuvant mechanism of liposomes is characterized by their ability to interact with APCs, enhancing the exposure of Ag and immunostimulators to the APCs by acting as delivery systems (*Tandrup Schmidt S. et al., 2016*). Liposomes are good candidate adjuvants since they are characterized by the key features of versatility and plasticity. Indeed, liposome composition can be tailored by the choice of lipid components with different charge, size and preparation method, while several different types of immunostimulators can be incorporated into the same adjuvant system in order to achieve the desired features. This versatility has also led to a vast amount of literature on different experimental liposomal formulations in combination with a wide range of immunostimulators (*Schwendener RA et al., 2014*). As a drawback, since the vaccines are likely to contain components from different molecular classes, the chemical and the physical stability of the different components should be considered, and the stability of liposome-adjuvanted vaccines should be carefully addressed early in the development of the vaccine. Intensive development efforts have resulted in a number of different liposome-based adjuvant candidates as part of marketed or clinically-tested vaccines against several different pathogens during the past 20 years. One example of a well-characterized liposome-based adjuvant is the cationic adjuvant formulation (CAF01), composed of the synthetic immune-stimulating mycobacterial cord factor glycolipid trehalose dibehenate (TDB) as immunomodulator, and the cationic membrane forming molecule dimethyl dioctadecylammonium (DDA), which has been shown to induce a powerful cell-mediated immune response and a strong Ab response, desirable for a high number of disease targets (*Agger EM et al., 2008*).

4.3.5 TLR agonists as promising vaccine adjuvant

TLRs are an evolutionarily conserved family of PPRs, which structurally belong to the IL1-R/TLR superfamily (TIR). In humans, 10 functional TLRs have been described, which can be subdivided according to their subcellular localization. TLR 1, 2, 4, 5, 6, and 10 are expressed on the cell surface, whereas TLR 3, 7, 8 and 9 are expressed in intracellular compartments such as

endosome and endoplasmatic reticulum. Instead, mice do not present a functional TLR10 due to an insertional mutagenesis of a retrovirus, but have additional TRLs (TRLs 11-13) beside those in common with humans (TRLs 1-9). A variety of innate immune cells as DCs, macrophages, mast cells, neutrophils, endothelial cells, and fibroblasts express TLRs, which play a central role in sensing danger signals and triggering the immune response. TLRs recognize groups of structurally similar and widely distributed molecules: TLR1, 2, 4 and 6 sense lipids and lipopeptides, TLR5 sense proteins, TLR3, 7, 8, 9 bind nucleic acids. Moreover, many synthetic and endogenous (self) ligands have been described for most TLRs (**Table 2**). The binding between a ligand and its TLR result in receptors conformational changes leading to the recruitment of crucial adaptor proteins, which signal thought different downstream pathways. The most common signaling pathway, which is shared by many TLRs, is that mediated by MyD88, inducing the activation of NF-κB and MAPK and leading to the secretion of pro-inflammatory cytokines, type I IFNs and chemokines (e.g. IFNs, TNF-α, IL-10, IL-6 and Il-12) (**Figure 9**). Thus, through different adapter proteins every TLR-ligand binding triggers the secretion of different molecules, which may modulate the Th1/Th2 balance influencing and regulating the type of adaptive immune responses elicited. This regulatory function of TLRs leads to the exciting consequence that DCs triggering trough different TLRs, results in the induction of distinct DCs responses and adaptive immunities, which prompted the study and the development of many different natural and synthetic TLR agonists. The potentiality of TLRs agonists as vaccine adjuvants has been extensively studied, leading to a long list of TLRs ligands successfully used as adjuvants. The following paragraph describes some of the most important TLRs ligands in pre-clinical or clinical stage of development as vaccine adjuvants.

TLR	Subcellular localization	Physiological ligands	Synthetic ligands
TLR1–TLR2	Plasma membrane	Triacylated lipopeptides	Pam_3CSK_4
TLR2	Plasma membrane	Peptidoglycan, phospholipomannan, tGPI-mucins, haemagglutinin, porins, lipoarabinomannan, glucuronoxylomannan, HMGB1	ND
TLR2–TLR6	Plasma membrane	Diacylated lipopeptides, LTA, zymosan	FSL1, MALP2, Pam_2CSK_4
TLR3	Endosome	dsRNA	PolyI:C
TLR4	Plasma membrane	LPS, VSV glycoprotein G, RSV fusion protein, MMTV envelope protein, mannan, glucuronoxylomannan, glycosylinositolphospholipids, HSP60, HSP70, fibrinogen, nickel, HMGB1	ND
TLR4–TLR6	Plasma membrane	OxLDL, amyloid-β fibrils	ND
TLR5	Plasma membrane	Flagellin	ND
TLR7	Endosome	ssRNA	Imidazoquinoline compounds: imiquimod, resiquimod, loxoribine
TLR8	Endosome	ssRNA	Resiquimod
TLR9	Endosome	DNA, haemozoin	CpG-A, CpG-B and CpG-C ODNs

Table 2. Localization TLRs and their physiological and synthetic ligands. Abbreviations: FSL1= *S*-(2,3-bispalmitoyloxypropyl)-CGDPKHSPKSF; HMGB1= high-mobility group box 1 protein; HSP= heat-shock protein; LTA= lipoteichoic acid; MALP2= macrophage-activating lipopeptide of 2 kDa; MMTV= mouse mammary tumour virus; ND= not determined; ODN= oligodeoxynucleotide; oxLDL= oxidized low-density lipoprotein; RSV= respiratory syncytial virus; tGPI-mucin= *Trypanosoma cruzi* glycosylphosphatidylinositol-anchored mucin-like glycoprotein; VSV= vesicular stomatitis virus (modified from *Lee CC et al., 2012*)

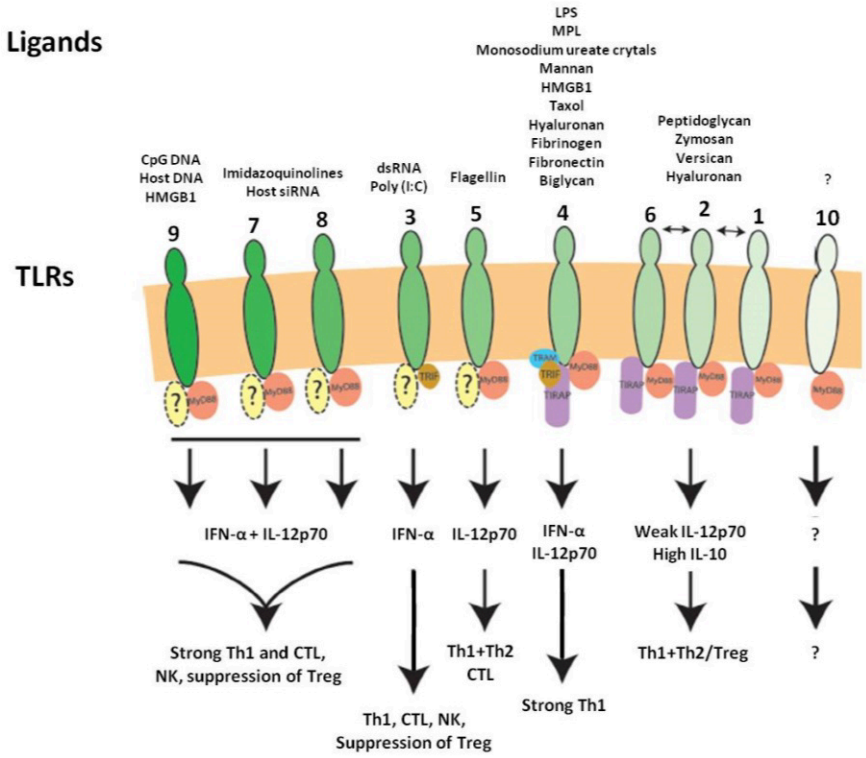

Figure 9. Regulation of adaptive immune responses by TLRs after interaction with their ligands. Triggering DCs through TLRs results in the induction of distinct adaptive immune responses (modified from *Pulendran B, 2004*).

TLR 2 and 4: Lipopolysaccharide and analogues

Bacterial products or synthetic mimics are potent stimulators of the innate immune system and most of these agents signal through TLRs. Multiple TLR2 agonists have been identified as molecules expressed by microbes and fungi, including natural and synthetic lipopeptides (e.g. *Mycoplasma fermenta),* peptidoglycans (PG, such as those from *S. aureus*), lipopolysaccharides (LPS) from various bacterial strains, and polysaccharides (e.g. zymosan). All these molecules, present in the bacterial cell wall could potentially serve as adjuvants due to their ability to induce immunity, but their toxicity make them unsuitable for human use. The most popular and widely studied among TLR ligands is the bacterial LPS, which is found in the outer membrane of gram negative bacteria and interacts with TLR4. LPS is known to be a potent inducer of macrophage activation, dendritic cell maturation and inflammatory cytokine production and, not surprisingly, has significant immune adjuvant activity. In particular, LPS stimulates robust IL-12 production from DCs *in vitro*, and thus it would be predicted to promote Th1-type responses *in vivo* (*Brightbil HDl et al., 1999*). However, despite its potency, LPS has been used only as an experimental adjuvant in animal studies because of its toxicity and pyrogenicity in humans. To bypass this issue, some less reactogenic LPS analogues have been obtained by modifying the Lipid A component of LPS, and have been explored as vaccine adjuvants. In particular, a non-

toxic derivative of the lipid A portion of LPS of *Salmonella minnesota*, namely Monophosphoryl Lipid A (MPL), was obtained by removing a phosphate group, the sugar moiety, and an ester-linked fatty acid group from Lipid A, resulting in a compound which is less toxic but retains the ability to activate the innate immunity by interaction with TLR4, and leads to activation of NF-κB signaling and production of pro-inflammatory cytokines and chemokines. Numerous clinical studies examining the adjuvant activity of MPL combined with vaccines targeting a wide variety of pathogens and tumor Ags have been conducted so far. In particular, MPL has been successfully used in a variety of proprietary adjuvant formulations developed by GlaxoSmithKline (GSK), with the aim of defining the best combination in which individual components can synergize to elicit a more robust immune response. Among these adjuvant systems, the most successful is AS04, were MPL is used in combination with aluminum hydroxide in an O/W emulsion. Data obtained with AS04 adjuvant showed that much of the adjuvant activity of this mixture can be attributed to the MPL component, resulting in increased Ab responses but at the same time significantly increased vaccine reactogenicity over Alum; AS04 induces robust Th1-type immune responses by promoting IL-2 and IFN-γ production, with an extremely safe profile (*Didierlaurent AM et al., 2009*). Two AS04-adjuvanted vaccines are already licensed for human use: the HPV vaccine (Cervarix, GSK Biologicals) and HBV vaccine (Fendrix, GSK Biologicals).

TLR3: poly I:C

TLR3 is triggered by double-stranded RNA (dsRNA) produced during the replication of most infectious viruses. The interaction of TLR3 with dsRNA initiates a TRIF-dependent signaling cascade that progresses through the activation of NF-κB and MAPKs, and culminates in the production of inflammatory cytokines and type I IFNs resulting in a Th1-polarized immune response that makes TLR3 agonists attractive adjuvant candidates for vaccines designed to combat intracellular pathogens and cancer. The archetypal TLR3 ligand, polyI:C (polyriboinosinic:polyribocytidylic acid) is a synthetic analog of dsRNA that interacts also with the cytoplasmic receptors melanoma differentiation-associated protein 5 (MDA-5) and the RIG-I. Because several tumor cell types express functional TLR3, MDA-5 and/or RIG-I, poly(I:C) have been shown to have a direct effect both on tumor and immune cells, acting as synthetic immunological danger signals capable of eliciting robust CMI and potent type I interferon responses and enhancing vaccine-induced anti-tumor immune responses, thus contributing to tumor elimination in animal models and patients (*Cheng YS and Xu F, 2010*). Supported by these results, poly-I:C-containing cancer vaccines are currently extensively under study in ongoing trials (*Ammi R et al., 2015*).

TLR5: flagellin

TLR5 is triggered by a region of the flagellin molecule expressed by nearly all motile bacteria. When used as an adjuvant, flagellin is typically fused to a recombinant vaccine Ag and, in that form, directly induces DC maturation, triggering the up-regulation of co-stimulatory signals and Ag-presenting molecules (CD80, CD83, CD86, MHC class II, TNFα, IL-8, IL-1β, CCL2, CCL5) (*Means TK et al., 2003*). Unlike other TLR agonists, flagellin tends to produce mixed Th1 and Th2 responses rather than strong Th1 responses (*Huleatt J et al., 2007*). Bacterial flagellins can also signal through inflammasomes that contain NLR family CARD domain-containing protein 4 (NLRC4) (*Miao EA and Warren SE, 2010*), although it is not known whether this pathway contributes to the adjuvant activity of flagellin.

TLR7/8: imiquimod (R-837) and resquimod (R-848)

TLRs 7 and 8 are phylogenetically and structurally related TLRs that recognize single-stranded RNA (ssRNA) sequences containing poly-U or GU-rich sequences. The synthetic ligands for these TLRs are a class of small molecules called imidazoquinolines, and among them the most studied are imiquimod (R-837) and resiquimod (R-848). R-837 is an imidazoquinoline amine analog to guanosine with potent anti-viral and anti-tumor activities. This ligand predominantly activates TLR7 in a MyD88-dependent way and leads to the induction of the transcription factor NF-κB, production of pro-inflammatory cytokines, such as IFN-α and IL-12, leading to the activation of both innate and acquired immunity (*Hemmi H et al., 2002*). R-848 is another guanosine derivative that triggers both TLR7 and 8, and exerts its adjuvant activity by activating DCs and B cells and induces the secretion of cytokines optimal for Th1 cell immunity, and Ab production (*Wagner L et al., 1999*). These TLR7/8 agonists have been investigated as potential vaccine adjuvants but they have been approved by the Food and Drug Administration (FDA) only for topical administration, although pre-clinical studies indicate that imidazoquinolines can improve both the magnitude and quality of Ag-specific T cell and Ab responses to co-administered Ags. Despite the approved clinical use of the topical imiquimod (Aldara® Imiquimod 5% cream; 3M, MN, USA), no vaccines using TLR7, TLR8 or TLR7/8 agonists have progressed beyond early-phase clinical studies thus far, since they showed dose limiting toxicity when given orally or intravenously before ever reaching efficacious concentrations.

TLR9: CpG-ODN

TLR9 detects the unmethylated CpG motifs present at high frequency in bacterial DNA. The recognition of CpG DNA by cells expressing TLR9 has a cascading effect on the immune system, leading to activation of immune responses characterized primarily by the production of pro-

inflammatory and Th1-biased cytokines (including IL-1, IL-6, TNFα, IFNγ, and IL-12). The immunostimulatory activity of bacterial DNA is mimicked by synthetic 18–25 base oligodeoxynucleotides (ODN) with optimized CpG motifs (CpG-ODN) that have been studied extensively as adjuvants, either soluble or formulated in nanoparticles or virus-like particles (*Jennings GT and Bachmann MF, 2009*). Preclinical studies demonstrate that CpG-ODN can enhance Ab responses (*Tross D et al., 2008)* and promote vaccine immunogenicity by improving Ag uptake by professional APC (particularly plasmacytoid DC), triggering the functional maturation of APC, and generating a cytokine/chemokines microenvironment supportive of Ag-specific immunity (*Klinman DM et al., 2009*). Their utility as vaccine adjuvants was evaluated in a number of clinical trials, whose results indicate that CpG-ODN improve Ag presentation and the generation of vaccine-specific cellular and humoral responses. Toxicity has not been observed in animal studies of CpG adjuvanted vaccines, and evidence from clinical trials indicates that CpG ODN are reasonably well tolerated when administered as vaccine adjuvants (*Scheiermann J and Klinman DM, 2014*).

Natural polymers

Among TLR agonists, natural polymers (NPs) originating from plants, animals, and microbes are emerging as promising adjuvants for human vaccines due to their unique set of advantagous features. Their most suitable properties are the high biocompatibility and good tolerability *in vivo*; NPs are indeed readily biodegradable and therefore unlikely to cause problems of long-term tissue deposit seen with Alum adjuvants. For many decades, NPs have been used as delivery systems and widely employed in different clinical settings for long-acting delivery of nucleotide, peptide, and protein therapeutics, showing to be biocompatible, biodegradable, non-toxic, non-immunogenic and non-inflammatory *per se*. Naturally derived polymers have also the advantage of being widely available and therefore cheap. A number of carbohydrate polymers have been demonstrated to have strong immunostimulating activity as vaccine adjuvants, successfully eliciting an immune response by involving several immune system players. By acting as DAMPs or PAMPs and interacting with TLRs, some NPs exert a direct effect on monocytes and DCs and induce their activation, resulting in long-lasting humoral and cellular immune responses (*Justin RA and Mallapragada SK, 2014*). Alternatively, when conjugated to the Ag, NPs can be phagocytosed and processed through proteasomes, leading to the activation of inflammasome pathway via secretion of IL-1β cytokine, or can directly interact with B cells and consequently trigger the immune cascade (**Figure 10**).

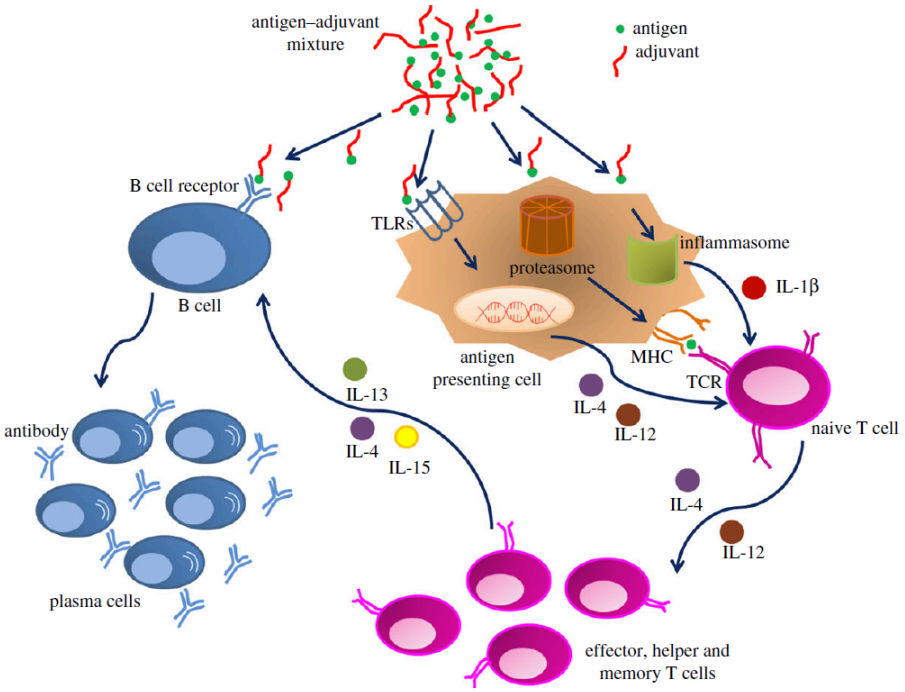

Figure 10. Possible mechanisms of action of polymeric adjuvants (from *Shakya AK and Nandakumar KS, 2012*)**.**

Ag can be loaded into polymers through several different methods that include entrapment, surface chemical conjugation, and physical adsorption. Thanks to their chemical and physical properties, NPs have a strong potential for functionalization and can be rationally modified by tailoring their molecular weight, surface charge, hydrophobicity, and degradation kinetics to improve their adjuvanticity. Indeed, they can be easily modified to reflect the properties of a pathogen and train the immune system to respond appropriately, or they can be tailored to incorporate additional immunomodulatory properties.

Many naturally derived polymeric compounds such as zymosan, chitosan, mannan, and dextrans are showing good promise as vaccine adjuvants, with several of these molecules already involved as vaccine adjuvants in human trials (*Shakya AK and Nandakumar KS, 2012; Wilson-Welder JH et al., 2009*). In particular, chitosan is a well-studied NP with immunostimulating, biodegradability, bioadhesion and penetration-enhancing properties (*Amidi M et al., 2010*). Chitosan is the deacetylated derivative of chitin, a linear polysaccharide found in the exoskeletons of crustaceans, and in the cell walls of fungi. One of the most relevant features of chitosan is the possibility to modulate its degree of deacetylation and the length of the polymer chain in order to change its physiochemical properties (*García-Fuentes M and Alonso MJ, 2012*). By combining its bioadhesive properties and its ability to open tight junctions in cell membranes, this polymer has been shown to improve transport of the Ag across mucosal membranes, and has therefore been

studied extensively as an oral or nasal delivery system for the Ag. Although the activity of chitosan is not fully understood, it has been demonstrated that it stimulates the immune system by activating the NLRP3 inflammasome, leading to potent IL-1β production and to the induction of a balanced Th1/Th2 response (*Neumann S et al., 2014; Wen ZS et al., 2011*).

Taken together, the large number of clinical trials involving TLRs 2, 3, 4, 7/8 and 9 agonists supports the conclusion that TLR ligands can be safe and effective vaccine adjuvants, with some of them already included in human vaccines licensed in the US and Europe. The peculiarity of different TLRs to trigger different cell types following stimulation with different ligands, thus influencing the nature of the immune response, is a great weapon that can be employed to investigate combinations of adjuvant molecules with huge advantages over use of a single agonist.

Despite the numerous compounds involved in pre-clinical and clinical studies, only few are currently approved as adjuvants for usage in humans (**Table 3**). So far, most of the licensed adjuvants are effective in enhancing Ab titers, but do not elicit strong Th1 and/or CTL responses. To overcome this limitation, the incorporation of agonists of TLRs and/or other innate immune receptors is emerging as an interesting strategy currently under investigation in new generation vaccines (*Reed SG, Orr MT et al., 2013*).

Adjuvants	Proposed mechanism of action	Immune response activated	Licensed vaccines	Reference
Alum	No depot effect NLRP3 activation in vivo? Independent of TLR signaling ↑ local cytokines and chemokines ↑ cell recruitment(eosinophils, monocytes, macrophages) ↑ Ag presentation	↑ Ab responses ↑ Th2 responses Poor TH1 responses	Many human vaccines (e.g., DTap, Hib, Hepatitis A and B)	Gavin et al. (2006), Franchi and Nùñez (2008), Kool et al. (2008a), McKee et al. (2009), Hutchison et al. (2012)
MF59	No depot effect NPLR3 independent but ASC-dependent Independent of TLR signaling but MyD88-dependent for Ab responses ↑ local cytokines and chemokines ↑ cell recruitment (neutrophils, macrophages, and monocytes) ↑ Ag uptake Activate muscle cells ↑ Ag-loaded neutrophils and monocytes in dLNs	Balanced Th1 and Th2 responses	Licensed for influenza vaccine (FLuad®), H5N1 pre-pandemic vaccine (Aflunov®), H1N1 pandemic vaccines (Focetria® and Celtura®)	Depuis et al. (1999), Mosca et al. (2008), Calabro et al. (2011), Ellebedy et al. (2011)
AS04	MPL signals through TLR4 to activate APCs ↑ local cytokines and chemokines ↑ Cell recruitment (DCs and monocytes) ↑ Ag-loaded DCs and monocytes in dLNs	↑ Ab responses ↑ Th1 responses	Licensed for human papilloma virus (HPV) (Cervarix™), hepatitis B virus (Fendrix®)	Didierlaurent et al. (2009)
AS03	Spatio-temporal co-localizantion with Ag Transient ↑ cytokines locally and in dLNs ↑ cell recruitment (granulocytes and monocytes) ↑ Ag-loaded monocytes in dLNs	↑ Ab responses ↑ Immune memory	Licensed for pandemic flu vaccine (Pandemrix®)	Morel et al. (2011)
Virosomes	Ag delivery vehicle Bind APCs and induce receptor-mediated endocytosis Escape endosomal degradation Ag presentation via MHC class II and MHC class I to CD4+ T cells and CD8+T cells respectively Immunopotentiator	↑ Ab responses ↑ CTL	Licensed for Inflexal®V and Invivac® influenza vaccine and hepatitis A vaccines (Epaxal®)	Glück et al. (1992), Bungener et al. (2002a,b), Khoshnejad et al. (2007)

Table 3. Features and application of adjuvants licensed for human use (modified from *Awate S. et al., 2013).*

Aim of the thesis

The emerging popularity of subunit vaccines, constituted by poorly immunogenic synthetic and recombinant antigens, has underlined the importance of discovering new and potent adjuvants able to enhance strong immune responses. Many different classes of adjuvants are currently under development in pre-clinical or clinical stage, but only a few are licensed for clinical use. Therefore, there is an increasing need for novel adjuvants capable of augmenting immunogenicity for both humoral and cell-mediated responses. The class of NPs is emerging as a promising alternative among adjuvant formulations. In particular, NPs, which can act as DAMPs or PAMPs, have been studied for many years since they offer the advantages conferred by their ability to interact with TLRs and, as vaccine adjuvants, have been shown to efficiently activate DCs and confer long-lasting immune responses without overt toxicity.

In our laboratory, a new promising vaccine adjuvant has been identified, called NPX for patent constraints, which is able to interact with two different TLRs. NPX is a biodegradable, biocompatible, non-toxic and non-inflammatory linear polysaccharide, which can be easily conjugated to virtually all types of antigens. Moreover, low molecular weight NPX derivatives behave as DAMP molecules, acting as TLRs agonists.

This project aimed at exploring the potentiality of NPX as an effective and safe adjuvant for protein-based vaccination studies. To this end, the model antigen OVA was chemically linked to NPX, and the resulting NPX-OVA bioconjugate was used for immunization of mice. Different vaccination schedules, concentrations of the conjugated antigen, and molecular weights of NPX were tested in order to assess the intrinsic features of NPX as adjuvant. Since the lack of animal studies or trials comparing multiple adjuvants with the same antigens is often noted as an impediment to adjuvant selection, a goal of this thesis was also to perform a comparison between NPX and different relevant commercial adjuvants, based on the same OVA antigen. Therefore, NPX adjuvant profile, properties, and efficacy were initially compared with those of Alum, the most widely used adjuvant in human vaccines, and then with other classes of commercial adjuvants such as Quil-A, Addavax, CFA/IFA, Chitosan, LPS, Montanide ISA50, and Montanide ISA 720. Finally, the last part of this thesis focused on a deeper investigation of NPX mechanisms of action by evaluating its biodistribution *in vivo* and its effect on immune cells at injection site and in the draining lymph nodes.

Materials and Methods

1. Mice and ethics statement

Six to eight week-old BALB/c (H-2^d) and C57BL/6 (H-2^b) female mice were purchased from Charles River Laboratories (Calco, Italy) or bred in IOV-IRCCS Specific Pathogen Free (SPF) animal facility. All mouse strains used for this study were housed in our SPF animal facility. Procedures involving animals and their care were in conformity with Institutional Guidelines (D.L. 26/2014, and subsequent implementing circulars), and experimental protocol was approved by the local "Organismo Preposto al Benessere Animale" (OPBA) of Padua University, and authorized by the Italian Ministry of Health (Authorization n.1249/2015-PR).

2. Cell lines

The following murine tumor cell lines were used: EL-4 (C57BL/6J, H-2^b, thymoma) and EG.7-OVA cells (EL-4 cells stably transfected by electroporation with the plasmid pAc-neo-OVA, which carries a complete copy of chicken OVA mRNA and the geneticin (G418) resistance gene). Cells were cultured in DMEM (EuroClone, Milan, Italy) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco BRL, Paisley, UK), 2 mM L-glutamine, 10 mM HEPES, 100 U/mL penicillin/streptomycin (all from Lonza, Verviers, Belgium) and 5 μM 2-βMercaptoethanol, hereafter referred to as complete medium. EG.7-OVA cell line was kept in culture in the presence of 0.4 mg/ml G418 antibiotic (Geneticin, Invitrogen, Milan, Italy) for transgene selection. Cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

3. Chemicals, reagents and commercial adjuvants

The model antigen EndoGrade® Endotoxin-free Ovalbumin (OVA) was purchased from Hyglos GmbH (Bernried am Starnberger See, Germany). Adjuvants LPS-EB Vaccigrade™, Chitosan Vaccigrade™, Quil-A®, AddaVax™ were supplied by InvivoGen (San Diego, USA). Imject™ Alum Adjuvant (Alum) was purchased from Thermo Scientific (Waltham, USA), complete and incomplete Freund's Adjuvant were from Sigma-Aldrich (St. Louis, USA) and Montanide™ ISA 51 and Montanide™ ISA 720 were purchased from Seppic (Puteaux, France).

4. Adjuvants and antigen preparation

The conjugation of NPX adjuvant to the C-terminus of OVA was performed by Professor G. Pasut, Department of Pharmaceutical Sciences, University of Padua (Italy), and resulted in a chemically stable bioconjugate hereafter called NPX-OVA. Both the nature of NPX and the specifics of its chemical conjugation to antigens are not reported in this thesis due to patent constraints.

Notably, the chemical ligation of antigens to NPX confers antigen solubility in water, and the absence of LPS contamination following conjugation process was checked using the commercial kit PYROGENT™ Gel Clot LAL Single Test Vial with a sensitivity of 0.125 EU/ml (Lonza).

OVA was dissolved in endotoxin free water and injected unconjugated, conjugated with NPX, mixed or emulsified with the different commercial adjuvants, according to manufacturer's instructions. Briefly, LPS-EB Vaccigrade™, Chitosan Vaccigrade™ and Quil-A® were mixed to the antigen at a working concentration of 25 µg/mouse, 100 µg/mouse and 10 µg/mouse in physiological solution, respectively. The other emulsion adjuvants were prepared in agitation (1400 rpm at RT) for at least 30 minutes. In particular, Imject™ Alum Adjuvant was added to antigen suspension in a final volume ratio 1:1, and the same procedure was used to prepare the emulsion of the antigen with Complete and Incomplete Freund's Adjuvant; AddaVax™, Montanide™ ISA 51, and Montanide™ ISA 720 were emulsified 50/50 (v/v) with the antigenic aqueous phase.

5. Immunization schedule and sera collection

Six to eight week-old female mice were immunized with OVA plus adjuvant in a final volume of 20 µl by intramuscular (i.m.) injection in the anterior tibialis muscles. Animals were vaccinated with different immunization schedules: either a single injection or a standard schedule, which consists in a prime injection at day 0 and two boosts at day 14 and 21, were performed with different antigen concentrations. The relative sera were collected at day 0, as basal control, before every subsequent immunization, and thereafter every month for until 1 year. Blood samples were obtained from the facial vein of anesthetized mice with isoflurane/oxygen, and sera were obtained after 30 minutes incubation at 37°C followed by centrifugation at 4000 rpm for 10 minutes and immediately processed or stored at -20° C until analysis.

6. ELISA

Individual sera from immunized mice were collected at different stages of the immunization schedule and were titrated for OVA-specific IgG and IgG subclasses (IgG_1, IgG_{2a}, and IgG_{2b}) by enzyme-linked immunosorbent assay (ELISA). Half area 96-well plates (Corning Life Sciences, NY, USA) were coated overnight at 4°C with 10 µg/ml OVA diluted in 0.005 M sodium carbonate/bicarbonate buffer (pH 9.6, Sigma-Aldrich) in a final volume of 60 µl/well. Plates were washed 5 times with PBS1X (100 µl/well, Sigma-Aldrich), and saturated for 2 hours at RT with 100 µl/well of PBS1X with 2% milk (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Two-fold serial dilutions of mice sera (from 1:50 to 1:3200) were added in duplicate to the plate (30 µl/well) and incubated for 1 hour at RT. Negative controls were milk 2% alone and sera at day 0

diluted 1:50 in milk 2%. The plates were washed 5 times with PBS 1X, and incubated for 1 hours at RT with the HRP-conjugated goat polyclonal anti-mouse IgG, IgG_1, IgG_{2a}, IgG_{2b} Abs purchased by Bethyl Laboratories (Montgomery, TX, USA), diluted 1:5000 in PBS 0.05% Tween®20 (Sigma-Aldrich). After another washing step in PBS 1X plates were dried and incubated with 60 µl/well of the substrate SIGMAFAST® OPD (o-phenylenediamine dihydrochloride, Sigma-Aldrich) dissolved in water. Following 5 minutes incubation in the dark at RT, the reaction was blocked adding 30 µl/well HCl 3N (Carlo Erba, Milan, Italy). Plates were immediately read with VICTOR X4 Multilabel Plate Reader spectrophotometer (Perkin Elmer Inc., Massachusetts, USA) at 490 nm. Quantification of IgG and IgG subclasses content was assessed by performing titration curves. Briefly, plates were coated with 5 µg/ml goat anti-mouse Polyvalent Immunoglobulins (Sigma-Aldrich) and then incubated for 1h at RT with 30 µl/well of sequential dilutions of IgG from mouse serum (Sigma-Aldrich) or previously purified and quantified IgG_1, IgG_{2a} and IgG_{2b} mAbs. The plate was then treated as described above, and the correlation between absorbance and IgG or IgG subclasses concentration was analyzed by linear regression analysis. Th1/Th2 index was obtained by calculating the IgG_{2a}/IgG_1 ratio using the mean value of IgG_1 and IgG_{2a} content (µg/ml) of sera collected at different time points from immunized mice.

7. Cytokine production

To define the Th1/Th2 profile induced by NPX-vaccination, BALB/c mice were vaccinated i.m. with 10 µg of OVA alone, conjugated with NPX, or emulsified with Alum with standard immunization schedule, while untreated mice were used as negative control (three mice per group). At day 30, mice were sacrificed and spleens removed. A total of 10^6 splenocytes/well were plated in triplicates in flat-bottom 96 well plates, and stimulated with 5 µg/ml of OVA protein, or medium alone (basal cytokine release), in a final volume of 200 µl of DMEM complete medium. Plates were maintained at 37°C and 5% CO_2, and supernatants were harvested at 72 h. Samples were analyzed for their cytokine content (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-13, IL-22, IFN-γ, TNF-α) with Luminex xMAP® technology (multiplexed fluorescent bead-based immunoassay, Luminex, TX, USA), using Mouse Th17 Multiplex Kit (Merck S.p.A., Milan, Italy), according to the manufacturer's instructions.

8. Mixed lymphocyte tumor cell culture (MLTC)

Spleens from mice immunized i.m. with 10 µg OVA either unconjugated, conjugated with NPX, mixed or emulsified with the other commercial adjuvants as described above, were collected at day 30, mashed onto 100 µm nylon filter (Falcon, Becton Dickinson) and washed in complete medium. MLTC cultures were set up by *in vitro* stimulation of $25x10^6$ splenocytes with $1x10^6$

irradiated (60 Gy) EG.7 OVA-expressing tumor cells. Cell cultures were maintained in 10 ml DMEM complete medium, in 25-cm^2 tissue culture flasks (Falcon) for 5 days at 37°C, 5% CO_2.

9. IFN-γ ELISA

Supernatants were collected from MLTC after 72 hours of incubation and tested in an ELISA in order to evaluate the production of IFN-γ by stimulated splenocytes. IFN-γ ELISA was performed in freshly collected supernatants using mouse IFN-γ microplate kit (R&D systems, Minneapolis, MN, USA) following manufacture's instruction.

10. Cromium (^{51}Cr) release assay of MLTC

The cytotoxic activity of MLTC was assessed in a 4h ^{51}Cr-release assay performed after 5 days of culture. EL-4 and EG.7-OVA were used as target cells. Briefly, EL-4 cells were pulsed either with 10 µM $OVA_{257-264}$ MHC class I peptide (SIINFEKL, JPT Peptide Technologies GmbH, Berlin, Germany) or with 10 µM of the unspecific peptide beta galactosidase$_{96-103}$ (DAPIYTNV, CRIBI Biotechnology Center, University of Padua, Italy). All target cells were labeled with 100 µCi ^{51}Cr for 1 h at 37°C, washed twice and added to the effectors cells plated in 1:3 serial dilutions, starting from an effector/target (E/T) ratio of 100:1. Triplicates for each assay condition were set in round-bottom 96 well plates (Sarstedt, Nümbrecht, Germany) in a final volume of 200 µl/well of DMEM 3% FBS. As negative control (spontaneous release), cells were incubated with DMEM 3% FBS alone, while for positive control (maximum release) cells were treated with 200 µl/well of DMEM 2% SDS. After a 4 h-incubation at 37°C and 5% CO_2, 30 µl of supernatants were transferred in a 96-well solid scintillator coated plate (LumaPlate-96, PerkinElmer), let dry overnight at RT and radioactivity was evaluated using TopCount NXT γ-ray counter (PerkinElmer). The percentage of specific lysis was calculated as follows:

$$\% \text{ specific lysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

11. SIINFEKL-specific $CD8^+$ T cells staining

One week after the last injection, spleens and lymph nodes were collected from C57BL/6 mice immunized with 1 µg following the standard schedule. Single cell suspensions were obtained by mincing the organs through a cell-strainer followed by washing with PBS. Blood lymphocytes were separated by centrifugation using Lympholyte (Cedarlane, Burlington, Canada) at 900 × g for 20 min, followed by two washes in PBS. In a V-bottomed 96-well plate, 1×10^6 lymphocytes were stained for identification of antigen-specific $CD8^+$ T cells with PerCP-Cy5.5 anti-mouse $CD8^+$

(clone 53-6.7, BD, Franklin Lakes, NJ, USA) and with R-PE-labeled H-2Kb/SIINFEKL Pentamer (Proimmune, Oxford, UK). Data were acquired using a FACSCanto or a FACSFortessa (BD, Franklin Lakes, NJ, USA) followed by analysis using the FlowJo v10 software (Tree Star Inc., Ashland, OR, USA).

12. Biotolerability analysis

To compare toxicity induced by NPX or by other adjuvants at the injection site, BALB/c mice were injected once i.m. with the model antigen OVA alone (10 μg/mice), chemically linked to NPX, emulsified with Alum, CFA/IFA, Addavax, ISA 51 and ISA 720 or simply mixed with LPS, Chitosan or Quil-A as already described (3 mice/group). Animals were sacrificed at different time points (6 hours and 1, 3, 7, and 14 days after immunization) and tibialis muscles were collected for histological analysis. Tissue specimens were fixed in 4% neutral-buffered formalin, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin (H&E), and observed in a blinded manner. The H&E staining was performed in collaboration with Melanoma and Sarcoma Diagnostics Section, Veneto Institute of Oncology IOV-IRCCS, Padua, Italy.

13. Detection of inflammatory cells and cytokines at the injection site

Quadriceps muscles from mice immunized with the standard schedule were harvested at different time points (6h, 24h, 48h) and weighted. Cell suspensions were obtained by digestion and homogenization using the gentleMACS Octo Dissociator and Skeletal Muscle Dissociation Kit (MACS, Miltenyi Biotec Inc., Auburn, CA, USA) following manufacturer's instructions. After digestion, supernatants were separated from cells suspension by centrifugation and tested in a U-plex customized plate (Meso Scale Diagnostics, Rockville, Maryland, USA) for their inflammatory cytokines content (IL-10, IL-12p70, IL1β, IL-6, IL5, KC/GRO, MCP-1, MIP-1α, TNF-α) following manufacturer's instructions. Cells obtained from digested muscles were centrifuged, filtered, enumerated and stained with combinations of the following Abs for the detection of inflammatory cells at injection site: MHC Class II (I-A/I-E) PerCP-Cy5.5 (clone M5/114.15.2), CD11c APC-Cy7 (clone N418), CD11b PerCp-Cy5.5 (clone M1/70), Ly6G PE (clone 1A8), F4/80 PE-Cy7 (clone BM8). All fluorescently labelled Abs were obtained from BioLegend (San Diego, CA, USA) and BD. Data acquisition was performed with FACSCanto or FACSFortessa (BD) followed by analysis using the FlowJo v10 software (Tree Star Inc.).

14. *In vivo* imaging and fluorescence microscopy analysis

To follow *in vivo* antigen biodistribution, OVA was covalently labeled with the fluorophore Cy5.5 (in collaboration with Professor G. Pasut), BALB/c mice were injected i.m. with a single dose of

OVACy5.5, NPX-OVACy5.5 or Alum emulsify with OVACy5.5. The *in vivo* total body scanning was performed at different time points (0, 4, 8, 24, 48, and 72 hours) on isoflurane/oxygen-anesthetized animals, using an MX2 scanner (ART, Canada). Biodistribution kinetics of the compounds was assessed by fluorescence optical imaging as total body scanning with a 670nm laser and a 693LP filter, spatial resolution/scan step was fixed at 1.5 mm, exposure time was 0.1 s, and laser power was automatically adjusted for each scan session. Constant region of interest was manually selected and the fluorescence signal was analyzed using the ART OPTIX-OptiView software (version 2.02.01). To finely display the antigen fluorescence in the draining lymph node following i.m. injection, draining inguinal lymph nodes were isolated 6 hours post-injection, placed in molds containing OCT medium and snap frozen with liquid nitrogen. Specimens were sectioned at 4 µm, stained with DAPI (BD) and fluorescent images were captured a 10x and 60x objectives on a the fluorescence microscope Leica DM4000 B LED (Leica Microsystems GmbH, Wetzlar, Germany).

15. *In vitro* digestion of NPX-OVA

To test whether the enzyme NPXase (Sigma-Aldrich) is able to digest NPX also in conjugation with OVA and whether this digestion results in the production of LMW pieces of NPX, the bioconjugate NPX-OVA was incubated *in vitro* with the NPXase at 37°C and the nature of the products was evaluated by High Performance Liquid Chromatography (HPLC) at different time points.

16. DCs stimulation *in vitro* and flow cytometry

NPX capability to stimulate DCs was tested *in vitro* using BMDCs. Tibias and femurs from BALB/c mice were collected, the content of bone marrow was flushed using a 2 ml syringe, and washed at 4°C in complete DMEM. Red blood cells were depleted with 1 mL of lysis buffer for 5 minutes at RT. Cells were washed again, filtered in a 100 µm cell strainer, diluted 1:2 in Trypan blue solution (Sigma-Aldrich) and counted. At day 0 cells were seeded in 6-well plate at a concentration of $0.8x10^6$ cells/well in 3 ml of complete medium supplemented with 20 ng/mL recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ, USA) and 100 ng/mL of murine IL-4 (Peprotech). At day 0 some cells were stimulated with 50 µg/ml of LMW NPX derivative (Cosmo Bio Co. LTD, Tokyo, Japan), NPX-OVA or NPX-OVA digested with NPXase. Digestion was performed as previously described by incubating NPX-OVA with NPXase overnight at 37°C. Others cells remained unstimulated and were considered as control immature DCs (iDCs). After 2 days, 2 ml of supernatant and cells were removed, cells recovered by centrifugation and added back to the dish with fresh medium

supplemented with GM-CSF and mIL-4, and stimuli where required. At day 6 some wells were stimulated with 1 µg/ml LPS (Sigma-Aldrich) as positive control for DCs maturation. Cells stimulated with LMW NPX derivative, NPX-OVA or NPX-OVA digested with NPXase were washed and the medium was replaced with fresh medium and stimuli as described at day 2. Unstimulated iDCs were harvested and analyzed for their expression of maturation markers by flow cytometry. Briefly, cells ($0.4x10^6$/sample) were resuspended in 50 µL FACS buffer (PBS containing 2% bovine serum albumin and 0.02% NaN_3, both from Sigma-Aldrich), and stained at 4°C for 20 minutes in the dark with PE-conjugated anti-mouse CD11c, CD40, CD80, CD86 (Miltenyi Biotec S.r.l., Calderara di Reno, Italy), and FITC-MHC Class II molecules (Becton Dickinson, Franklin Lakes, NJ). After staining cells were washed, resuspended in 250 µl of PBS acquired with a flow cytometer FACSCalibur flow cytometer (Becton Dickinson) and analyzed as previously described. At day 9, stimulated cells were harvested and analyzed for the expression of maturation markers as already described.

17. Statistical analysis

Results were analyzed for statistical significance by using Student *t* test corrected for multiple comparisons using the Holm-Šídák method (P= multiplicity adjusted P values, *= P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001). All statistical analysis were performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA).

Results

1. Assessment of humoral immune responses induced by NPX

1.1 NPX induces highly efficient humoral immune responses

Previous preliminary data obtained in the laboratory, had shown that conjugation to NPX of different exogenous proteins led to elicit strong Ab responses, in the absence of added adjuvants. Therefore, to carry out an in depth analysis of the phenomenon, we decided to focus on a model antigen, namely ovalbumin, which has been always widely employed in vaccination and immunization studies. In this regard, to assess the effects of immunization with the NPX-OVA bioconjugate *in vivo*, the amount and quality of antigen-specific humoral response induced by NPX was compared to that obtained with Alum-adjuvanted vaccination. BALB/c mice were injected i.m. at day 0, 14, and 21 (hereafter referred to as standard schedule) with 10 μg of the model antigen OVA either unconjugated, conjugated to NPX or emulsified with Alum. We focused on the i.m. route of administration, since it is the most common and suitable among vaccination routes and, at the same time, allows a comparative analysis with other commercial adjuvants.

Adjuvanticity of NPX and Alum was evaluated in terms of quantity, quality and persistence of the antigen-specific humoral immune responses induced. OVA-specific total IgG, and IgG_1, IgG_{2a}, IgG_{2b} subclass amounts were measured and monitored over time. Results obtained at day 30 after 3 rounds of immunization (**Figure 11A**), showed that the protein alone induced only a negligible Ab response, which became evident and detectable upon Alum addition. Conversely, animals immunized with NPX adjuvant showed a very robust and strong anti-OVA humoral response, with highly increased IgG production in comparison to Alum. When IgG subclasses were analyzed, NPX turned out not only to induce significantly higher IgG_1 amounts, but differently from Alum was also able to elicit the production of IgG_{2a} and IgG_{2b} subclasses. Kinetics obtained by monitoring over time the IgG production in sera of immunized mice, showed how the NPX-induced humoral response was long-lasting, reaching a peak after the third injection (day 30) and remaining detectable for 1 year after priming. Notably, also IgG_{2a} and IgG_{2b} Abs were still detectable 1 year after the first injection of NPX-OVA bioconjugate (**Figure 11B**).

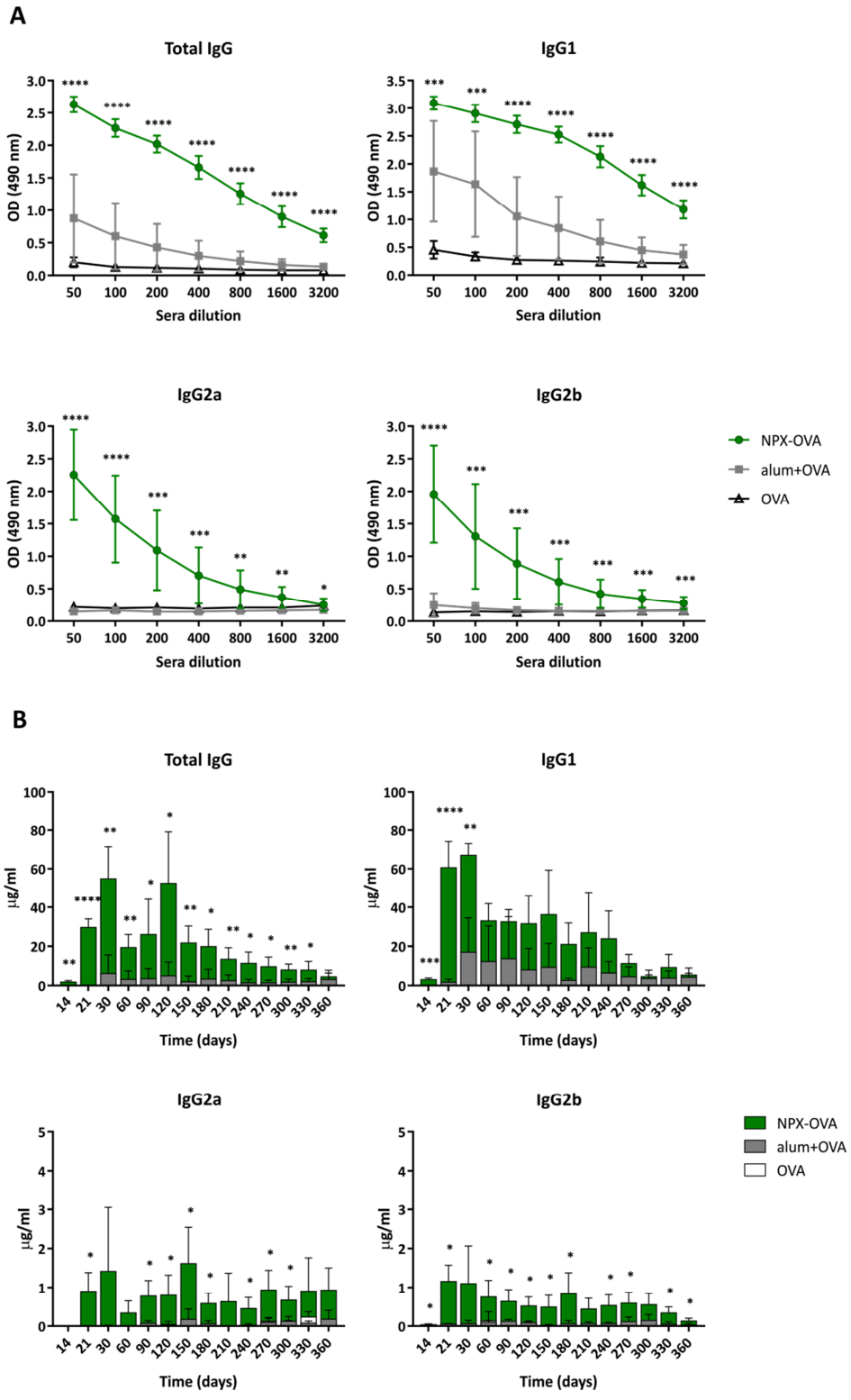

Figure 11. Total IgG and IgG subclasses content in sera of vaccinated BALB/c mice. BALB/c mice were immunized i.m. with 10 µg of OVA alone, conjugated to NPX or emulsified with Alum following the standard schedule. IgG and IgG subclasses content was detected by ELISA test. **A)** IgG contents detected at day 30 (n= 12 mice/group). **B)** Kinetics of IgGs concentration detected in sera of immunized mice over a period of 1 year (n=6 mice/group). Data were analyzed by multiple t-test; only statistics for Alum-injected *vs.* NPX-OVA group are reported [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

1.2 NPX adjuvanticity requires chemical conjugation of the antigen

To evaluate the role played by chemical conjugation to the antigen in NPX adjuvanticity, humoral responses were evaluated in mice immunized with OVA extemporaneously mixed with NPX (NPX+OVA), and compared with the other groups. Quantifications of whole IgG and related subclasses in sera of BALB/c mice immunized with the standard schedule, showed that NPX simply mixed with OVA entirely loses its adjuvant effect. IgGs production in mice immunized with NPX+OVA was indeed comparable to that obtained after immunization with OVA alone, clearly indicating that the covalent binding of NPX to the antigen is required and necessary for NPX to exert its adjuvant effect (**Figure 12**).

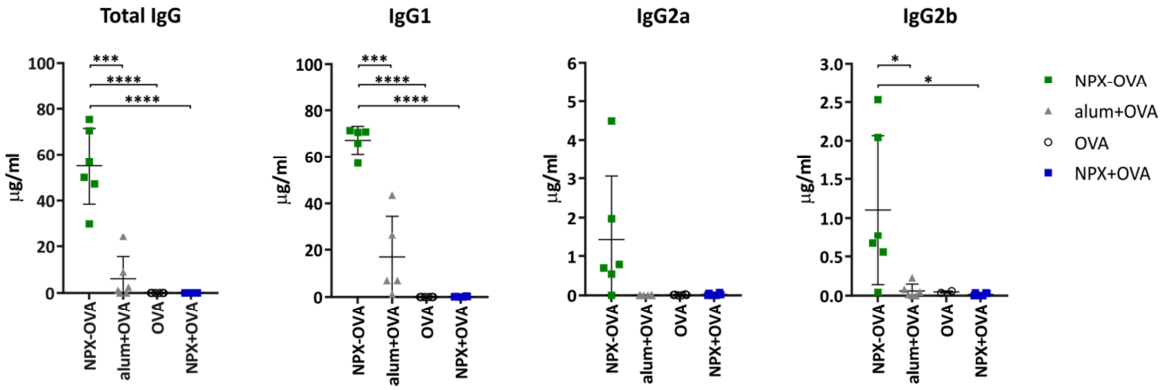

Figure 12. Quantification of total IgG and IgG subclasses production by vaccinated BALB/c mice at day 30. BALB/c mice were immunized i.m. with 10 µg of OVA alone, emulsified with Alum, chemically conjugated to NPX (NPX-OVA), or extemporaneously mixed with NPX (NPX+OVA), following the standard schedule. IgG and IgG subclasses content was quantified by ELISA (n=6 mice/group). Data were analyzed using multiple t-test [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

1.3 NPX does not require antigenic boosts and allows antigen dose sparing

To further investigate the potentiality of NPX, we challenged it for one of the most wanted feature of an ideal adjuvant: dose sparing. With the aim of reducing antigen dose and number of injections, we applied a single-dose immunization schedule where mice were injected only once with 10 µg of OVA. Thereafter, mice humoral response was monitored for one year to evaluate the kinetics of IgGs production. Such long-term follow up disclosed that NPX immunization was able to induce a long-lasting humoral response also with a single dose; this completely diverged from Alum, which showed to be unable to elicit a detectable humoral response after a single inoculation, but required repeated immunization steps (**Figure 13A**). Further analysis of IgG subclasses performed 30 days after vaccination showed that NPX-induced humoral response was mainly due to IgG_1, although also a low amount of IgG_{2a} and IgG_{2b} was detected (**Figure 13B**).

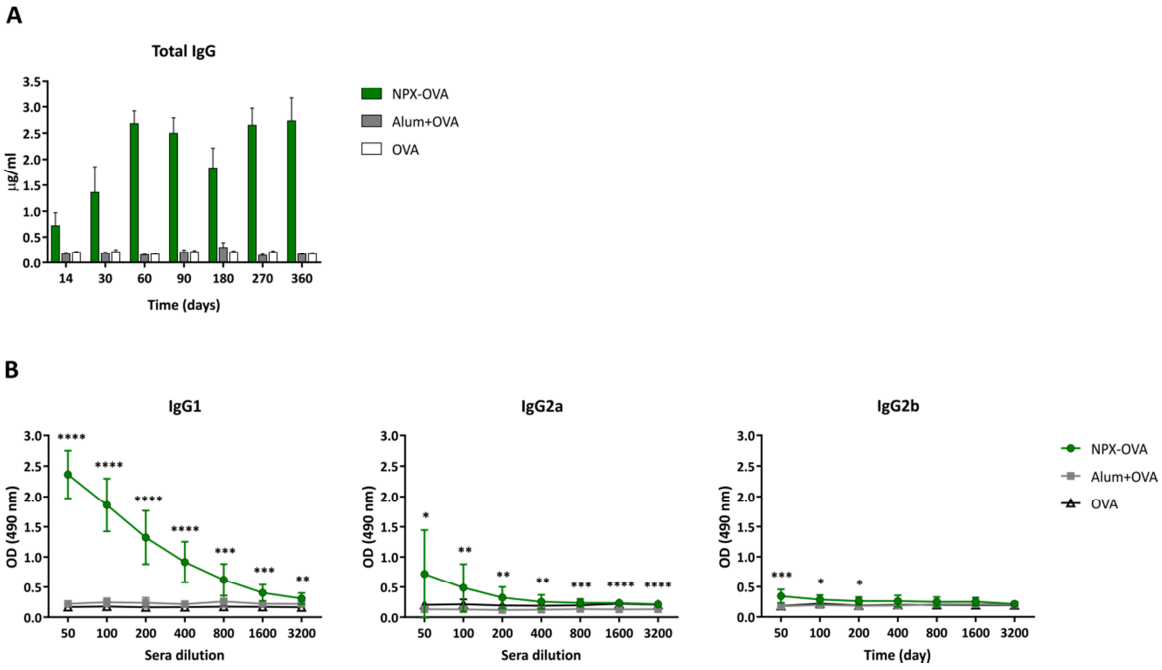

Figure 13. Total IgG and IgG subclasses content detected in sera from BALB/c mice immunized with a single injection. BALB/c mice were immunized i.m. with a single injection of 10 µg of OVA alone, conjugated to NPX or emulsified with Alum (n=6 mice/group). Data were analyzed by multiple t-test [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated]. **A)** Kinetics of total IgGs amount quantified up to 1 year after injection. NPX-OVA group *vs.* Alum group = ****, NPX-OVA group *vs.* OVA group = ****. **B)** IgGs subclasses content evaluated by ELISA 30 days after injection.

Since NPX appears efficient in inducing a humoral response after a single injection of the antigen, we tested its adjuvanticity also with low antigen concentrations. OVA was titrated and injected at the dose of 0.1, 1 or 10 µg in BALB/c mice following the standard schedule, and total IgGs production was evaluated. Results obtained in mice immunized with 0.1 µg of OVA revealed that NPX, differently from Alum, succeeded in inducing a detectable production of IgG even with such low antigen dose, and already after the second injection. This IgG production increased with the antigen dose, being already detectable at day 14 in animals receiving 1 µg of OVA and reaching, at day 30 with the same dosage, an amount of IgG that was comparable to that obtained with 10 µg of OVA; these results demonstrate that NPX acts as a powerful adjuvant capable of fostering strong humoral responses even against very low antigen concentrations. Conversely, Alum failed in inducing IgG production when injected with 0.1 and 1 µg of OVA, and proved to be efficient only after two injection of 10 µg of OVA (**Figure 14**).

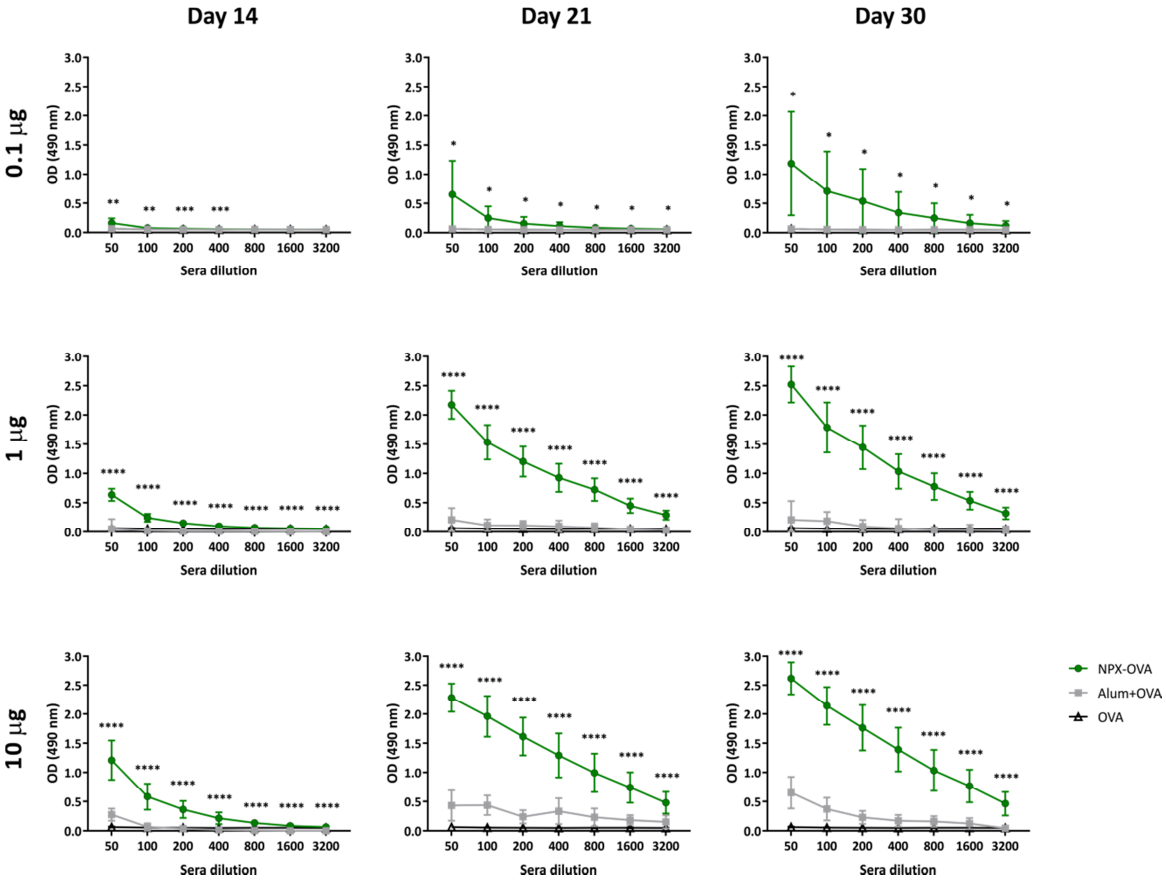

Figure 14. Total IgGs detected at different time points in sera from BALB/c mice immunized with different concentrations of OVA. BALB/c mice were immunized i.m. following the standard schedule with either 0.1, 1 or 10 µg of OVA alone, conjugated with NPX or emulsified with Alum. Total IgGs amount was evaluated by ELISA after each round of immunization (n=6 mice/group). Data were analyzed by multiple t-test; only statistics for Alum-injected *vs.* NPX-OVA group are reported [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

1.4 NPX adjuvanticity relies on its molecular size

Since it is known from literature that different molecular sizes of NPX exert different functions and have different biological effects, we asked whether NPX molecular weight might also influence its adjuvant activity. To answer this question, different molecular weight NPX (500, 50, and 15 kDa) were conjugated to OVA and injected in mice in comparison to our standard NPX-OVA bioconjugate, which derives from a 200 kDa NPX. **Figure 15A** shows that, after a single priming injection (day 14), only 200 kDa NPX was able to induce an IgG response in mice immunized with 10 µg of OVA, while similar levels of IgG were reached only after two injections of OVA conjugated to 50 kDa NPX. Notably and as confirmed by quantification analysis (**Figure 15B**), large-sized NPX showed to induce only a low production of IgGs, whereas the 15 kDa NPX was quite inefficient in eliciting an OVA-specific humoral response even after 3 doses. Thus, these experiments demonstrated that the NPX adjuvant effect depends on its molecular size, and prompted us to use the 200 kDa NPX as a standard in all subsequent experiments.

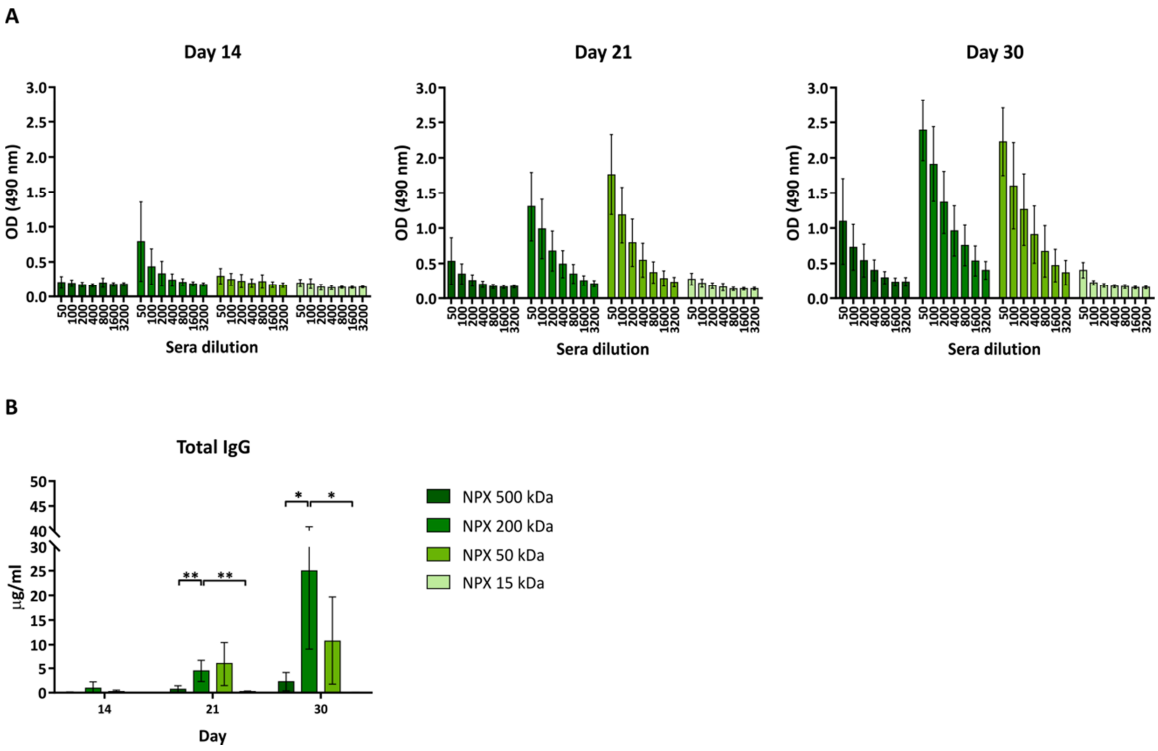

Figure 15. Total IgG content detected at different time points in sera of BALB/c vaccinated with OVA conjugated to NPX of different molecular weight. BALB/c mice were immunized i.m. following the standard schedule with 10 µg of OVA conjugated either with 500, 200, 50 or 15 kDa-sized NPX (n=4 mice/group). **A)** Total IgG detected at different time points by ELISA. **B)** Quantification of total IgGs at different time points. Data were analyzed by multiple t-test [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

1.5 NPX adjuvanticity is not strain-dependent

Since all previous immunization experiments had been carried out in the BALB/c mouse strain, we considered the possibility to confirm the adjuvant effect of NPX in a different immunocompetent strain routinely employed in laboratory, namely C57BL/6 mice. Results from a standard immunization protocol revealed that NPX-OVA induced comparable OVA-specific IgG levels between the two different mouse strains (**Figure 16**). This finding is even more relevant whether we consider that Alum failed to elicit an Ab response in C57BL/6 mice while being active in BALB/c animals, in agreement with several studies that highlighted differences in the immunological response of these two strains. In particular, BALB/c mice and C57BL/6 are designated as Th2 and Th1 type responders respectively, based on their cytokine profiles, and responses to infectious challenges and immunizations (*Busch RA et al., 2016*).

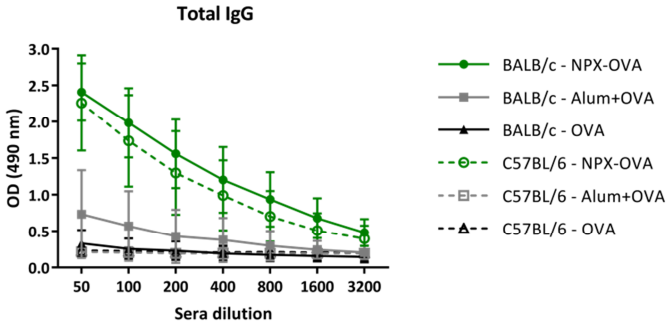

Figure 16. Total IgG content detected at day 30 in sera of BALB/c and C57BL/6 immunized mice. BALB/c and C57BL/6 mice were immunized i.m. with 10 µg OVA alone, conjugated to NPX or emulsified with Alum following the standard schedule. Total IgG amount in sera was evaluated at day 30 by ELISA test (n=12 mice/group).

1.6 NPX performs efficiently when compared to other adjuvants

Taken together, previous data demonstrate the superiority of NPX adjuvanticity over Alum, since NPX-based vaccination requires not only lower number of doses but also lower amounts of antigen per dose to induce robust humoral responses. Moreover, the NPX-induced Ab response is long-lasting and, differently from Alum, characterized also by the production of IgG_{2a} and IgG_{2b} subclasses.

Given these encouraging results, we planned to compare NPX-induced humoral response with that generated by other adjuvants. For this purpose, different commercially available adjuvants were selected from the most common adjuvant classes, and compared to NPX either using the standard schedule or a single injection, and also employing different mouse strains (**Figure 17**). Analysis of humoral responses after a single priming in BALB/c mice, revealed that NPX-induced IgG production was only lower than that induced by CFA/IFA adjuvant, and comparable to that produced by Montanide ISA 51 and ISA 720. Interestingly, with this protocol NPX showed to be always superior to all the other adjuvants tested (LPS, Chitosan, Quil-A, Alum and Addavax), which appeared indeed unable to elicit any detectable IgG production after a single antigen inoculation (**Figure 17A**). Following immunization with the standard schedule, NPX-induced IgGs approached the levels reached by CFA/IFA and the two Montanide versions, being however still slightly higher than values achieved with the other adjuvant tested (**Figure 17B**). The superiority of the humoral response induced by NPX in comparison to all the adjuvant tested, except CFA/IFA, was even more evident in C57BL/6 mice where lower IgGs production is generally observable (**Figure 17C**). Indeed, in this mouse strain NPX-stimulated Ab levels were very close to those observed in CFA/IFA-treated mice, and outperformed results gained with the other adjuvants.

A time-course quantification of total IgGs carried out in BALB/c mice, disclosed that the IgG production pattern of all tested adjuvants showed a peak at day 30 after the first injection, and a progressive slow decrease during the subsequent five months (**Figure 17D**). Taken together, these comparative experiments evidenced that the adjuvant effect of NPX is at least comparable to that exerted by most of the other experimental adjuvants tested, but largely outperforms the activity of Alum and Addavax, which are the only adjuvants approved for human use of this group.

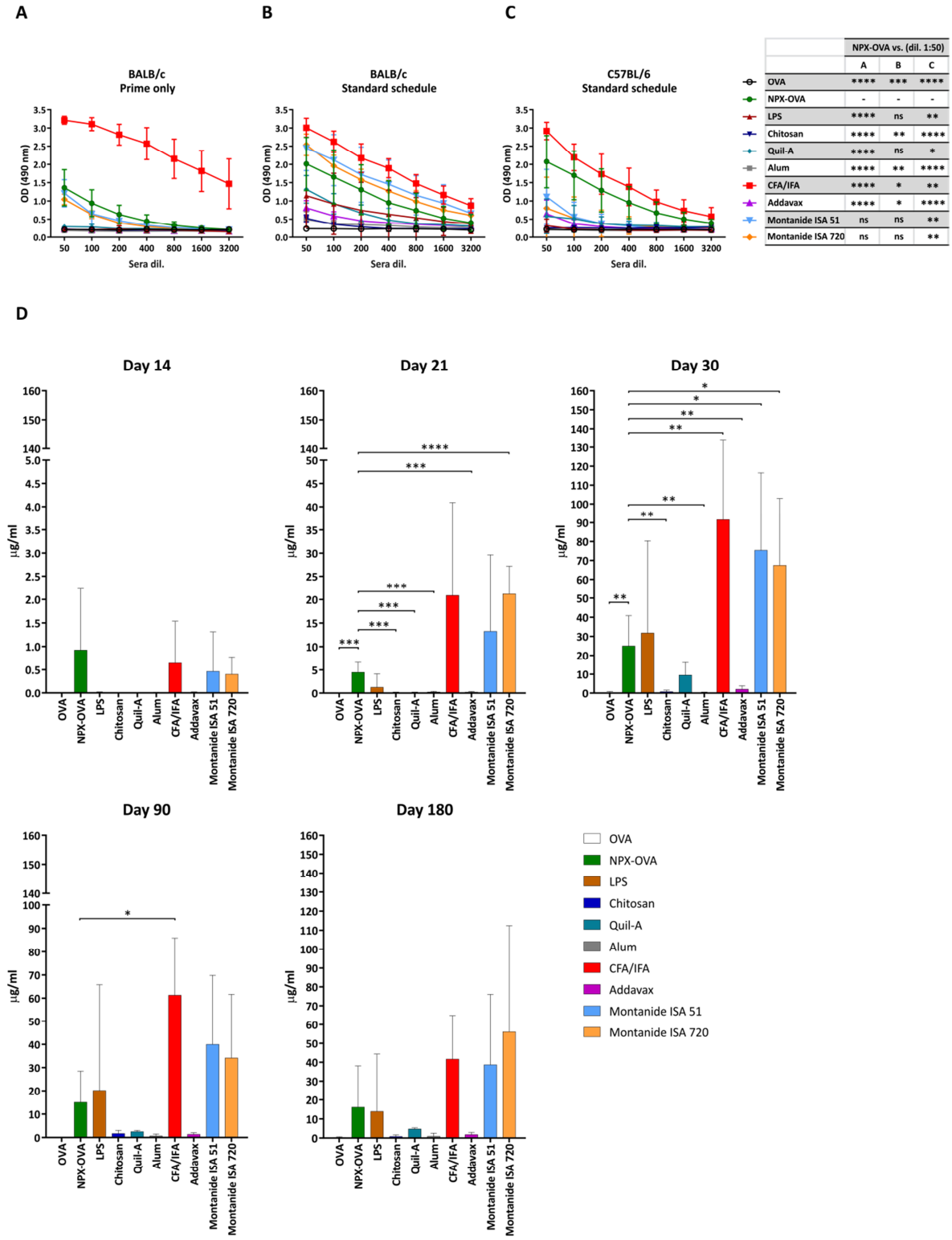

Figure 17. Total IgG content in sera of mice vaccinated with 10 µg of OVA injected alone or in combination with different commercial adjuvants. A-C) BALB/c (A and B) and C57BL/6 (C) mice were immunized i.m. with 10 µg OVA alone, conjugated to NPX or mixed or emulsified with different commercial adjuvants (LPS, Chitosan, Quil-A, Alum, CFA/IFA, Addavax, Montanide ISA 51 and Montanide ISA 720). Immunization protocols consisted in a single injection (A) or in 3 doses (standard schedule, B and C), and total IgGs amount was detected by ELISA at day 30. Multiple t-test was used to compare NPX-OVA with other adjuvants [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, ns = not statistically significant (p<0.05)]. Only statistics at sera dil. 1:50 are reported in the legend. **D)** Quantification of total

IgG contents measured 14, 21, 30, 90, 180 days after the first injection in sera of BALB/c mice immunized with the standard protocol (n=12 mice/group).

2. Evaluation of the Th1/Th2 polarization of the immune response induced by NPX

2.1 NPX stimulates a more balanced Th1/Th2 humoral response

Evaluation of how novel adjuvants modulates Th1/Th2-type immune response is a necessary requirement in vaccine research. In this regard, the quantity and the variety of the IgG subclasses elicited by an adjuvant is suggestive of the differential Th cell polarization it promotes. Thus, the strong ability of NPX to induce Abs of various subclasses of IgG, prompted us to evaluate the Th1/Th2 balance of such immune response. As currently held, Th1-type immune response is characterized by production of specific cytokines such as IFN-γ and IL-2, and is associated with the induction of cell-mediated immunity and the production of IgG_{2a} subclass in mice. On the other hand, Th2-type immune response relies on the secretion of IL-4, IL-5 and IL-13, and is associated with humoral immunity and a preferential development of IgG_1 isotype of mouse IgG. To get an idea of the Th1/Th2 polarization stimulated by NPX, the IgG_{2a}/IgG_1 ratio was determined along a period of 1 year. Results obtained from this calculation confirmed that Alum induced a Th2-skewed immune response, since the IgG_1 subclass was clearly dominant over the IgG_{2a} isotype. Conversely, NPX showed to stimulate a more balanced IgG_{2a}/IgG_1 humoral response, although the Th2-component remained dominant (**Figure 18**).

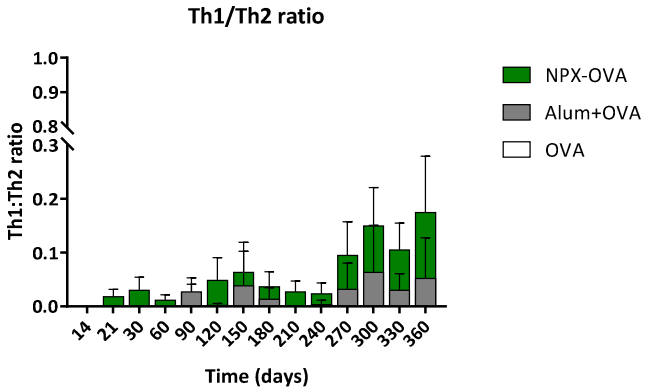

Figure 18. Th1/Th2 ratio in sera from vaccinated BALB/c mice. The Th1/Th2 ratio was obtained calculating the IgG_{2a}/IgG_1 ratio using the mean value of IgG_1 and IgG_{2a} content (µg/ml) of sera collected for 1 year from BALB/c mice immunized i.m. with 10 µg of OVA conjugated to NPX or emulsified with Alum, following the standard schedule (n= 6 mice/group).

2.2 NPX induces both Th1 and Th2 cytokines

IgG subclasses content suggested that i.m. administration of NPX-OVA induced both Th1 and Th2 immune responses, whereas Alum resulted in a Th2-skewed immunological reaction. To investigate this aspect more in depth and because cytokines secreted by T cells play a key role in the modulation of immune response, we decided to directly assess the cytokines induced by immunization with either NPX or Alum. To this end, spleens from BALB/c mice vaccinated i.m. according to our standard vaccination protocol with 10 µg of OVA unconjugated, conjugated with NPX or emulsified with Alum, were collected at day 30. Cell suspensions were stimulated *in vitro* with 5 µg/ml of OVA, and supernatants were harvested after 72 h of incubation and tested for their cytokines content by evaluating a wide range of Th1/Th2/Th17 cytokines (IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-13, and IL-22). The control group was represented by non-vaccinated BALB/c mice (**Figure 19**). Splenocytes of mice immunized with Alum produced typical Th2- and Th17-type cytokines, namely IL-5 and IL-6. Spleen cells from NPX-vaccinated mice disclosed detectable levels of all the cytokines tested; interestingly, NPX elicited slightly higher amounts of the Th1-type cytokines IFN-γ and IL-2 in comparison to Alum. Although spleen cells from non-vaccinated mice produced detectable amounts of IL-2, demonstrating that IL-2 production was not exquisitely OVA-specific, data about IFN-γ suggest that NPX might also stimulate the differentiation and expansion of CTL effectors. Nonetheless, no statistical difference was observed between the two adjuvants in the production of Th2- and Th17-type cytokines (IL-4, IL5, IL-6, IL-13, IL-22), thus confirming that NPX, similarly to Alum, stimulates also a strong Th2-type immune response.

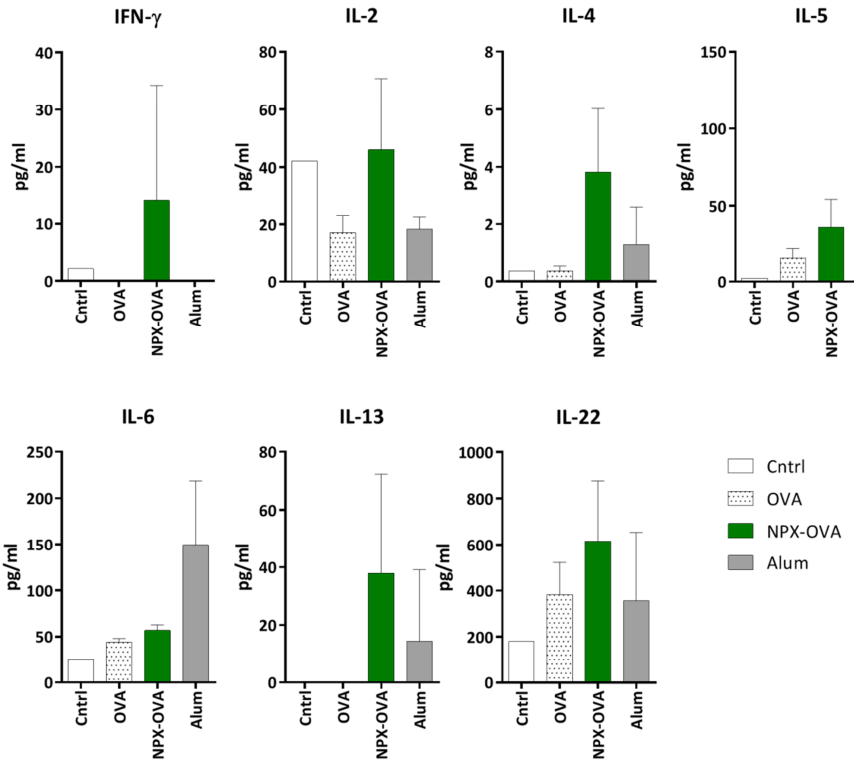

Figure 19. Cytokine production by splenocytes collected at day 30 from vaccinated BALB/c mice. Splenocytes were collected at day 30 from BALB/c mice immunized i.m. with 10 µg of OVA, NPX-OVA or OVA emulsified in Alum (n=3 mice/group, cntrl n=1). Splenocytes were restimulated for 72 h with 5 µg/ml of OVA protein. Cytokine content in cell supernatants was assessed with Luminex technology following manufacturer's instructions. Data were analyzed using multiple t-test [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

3. Assessment of cellular immune responses induced by NPX

3.1 NPX stimulates OVA-specific cytotoxic response

The production of detectable amount of IFN-γ by splenocytes of mice immunized with NPX, prompted us to study the potential ability of the adjuvant to stimulate a cell-mediated immunity. The ability to induce also cellular immune responses indeed, is a valuable feature for an adjuvant since cytotoxic T cells are major players in fighting against intracellular pathogens and cancer. To assess whether NPX, beside stimulating a strong humoral response, is also able to evoke cell-mediated reactions, splenocytes of C57BL/6 mice immunized i.m. with 10 µg of OVA alone, emulsified in Alum or conjugated to NPX, were isolated. Following *in vitro* stimulation of spleen cells with syngenic EG.7-OVA tumor cells in MLTC cultures, the potentiality of effector populations to specifically kill OVA-expressing target cells was determined by a ^{51}Cr release assay. **Figure 20** reports the percentages of MLTC specific lysis against the three target cells (EG.7-OVA, EL-4 + SIINFEKL and EL-4 + beta-gal), obtained at different effector:target (E:T) ratios. Results showed that stimulated splenocytes from the mice immunized with NPX-OVA were the most cytotoxic among the groups, against both EG.7-OVA and EL-4 + SIINFEKL cells, thus indicating the induction of OVA-directed CTL activity by NPX. Differently, Alum-adjuvanted vaccine showed to induce only negligible cytotoxicity levels to EL-4 cells pulsed with SIINFEKL, while no lysis was registered against EG.7-OVA cells. Notably, in all cases the lytic activity detected against EL-4 cells pulsed with the beta-galactosidase (beta-gal) unspecific peptide was practically absent, thus confirming the fine specificity of T cell cytotoxicity against OVA.

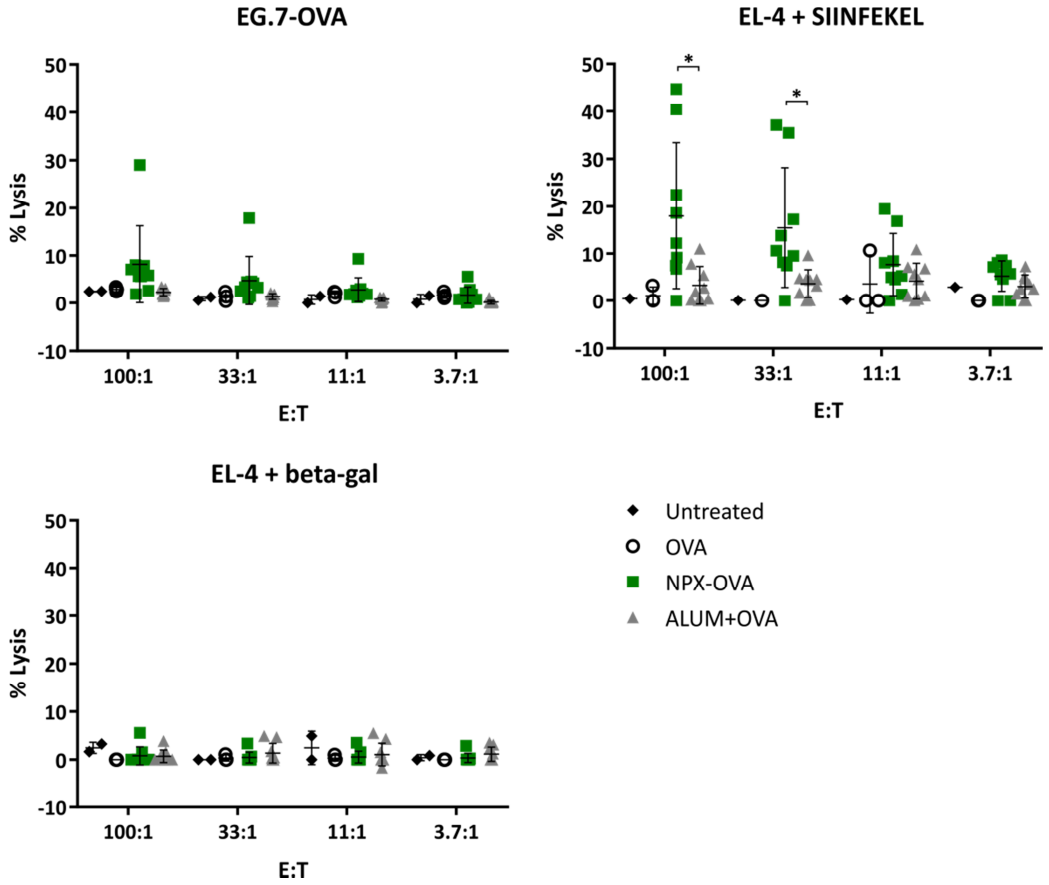

Figure 20. Cytotoxic T cell responses of vaccinated C57BL/6 mice against different target cell lines. Spleen cells of vaccinated C57BL/6 mice were restimulated *in vitro* at day 30 with EG.7-OVA cells, and evaluated 5 days later for their OVA-specific lytic activity against target cell lines by ^{51}Cr release assay. Each graph represents the percentages of lysis at different E:T ratios (T: target cells; E: effector cells) against EG.7-OVA, EL-4 + SIINFEKL or EL-4 + beta-gal target cells. Each symbol represents a single mouse, and bars indicate mean values ± SD (n=9 mice/group). Data were analyzed by multiple t-test; only statistics for Alum-injected vs. NPX-OVA group are reported [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

These findings suggested that vaccination with NPX-OVA might also elicit cellular immune responses in immunized mice, and prompted us to further investigate this aspect. Therefore, the presence of SIINFEKL-specific CD8$^+$ T cells was evaluated by pentamer staining of blood, lymph nodes and spleens from immunized mice collected at day 30 after the first injection. No SINFEKL-specific CD8$^+$ cells were identified in blood of any of the analyzed groups. Similarly, the number of SIINFEKL-specific CD8$^+$ cells enumerated both in lymph nodes and spleens from NPX-immunized mice was not significantly higher than values detected in the other groups, leading to the conclusion that immunization with 1 µg of OVA conjugated with NPX is not sufficient to induce the production of SIINFEKL-specific CD8$^+$ lymphocytes (**Figure 21**).

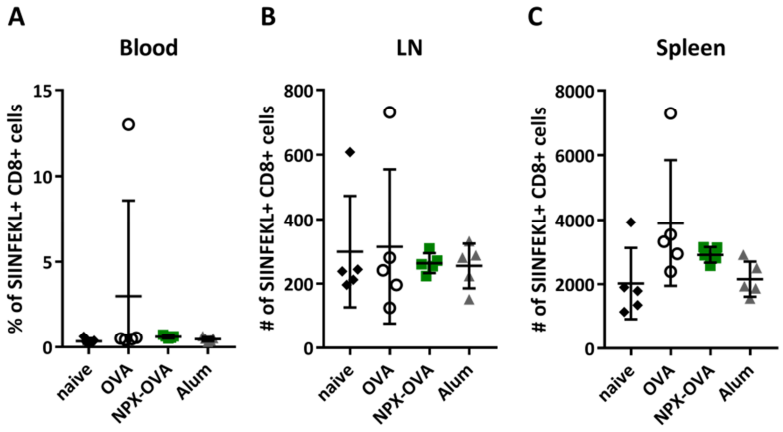

Figure 21. SIINFEKL-specific CD8$^+$ cells detected in organs of C57BL/6 immunized mice. Blood (**A**), lymph nodes (**B**) and spleens (**C**) were collected at day 30 from C57BL/6 mice immunized with 1 µg OVA following the standard schedule; cells were stained with anti-CD8$^+$ Ab and H-2Kb/SIINFEKL pentamer and analyzed by flow cytometry. Each symbol represents a single mouse, and bars indicate mean values ± SD (n=5). Data were analyzed by multiple t-test [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

3.2 NPX is not inferior to other adjuvants in inducing cellular immunity

Subsequently, we decided to compare the lytic activity stimulated by NPX with that induced by other adjuvants already known to generate potent cytotoxic responses. ^{51}Cr release assays were performed as previously described, using 5 days-stimulated splenocytes from C57BL/6 mice immunized with OVA alone or adjuvated with NPX, Alum, LPS, Chitosan, Quil-A, CFA/IFA Addavax and Montanide ISA 51 and ISA 720. Among them, CFA/IFA, Addavax, LPS, Montanide, Quil-A, are described in literature as Th1-stimulating adjuvants. Results obtained from repeated experiments were in agreement with literature, and showed the induction of cytotoxicity against EL-4 + SIINFEKL and EG.7-OVA target cells by Quil-A, CFA/IFA, Addavax, and Montanide ISA 720. Nonetheless, as previously reported also the immunization with NPX-OVA led to the induction of specific lysis against EL-4 + SIINFEKL and EG.7-OVA target cells, with an efficiency similar to the responses registered with well-known Th1-skewing adjuvants (**Figure 22**).

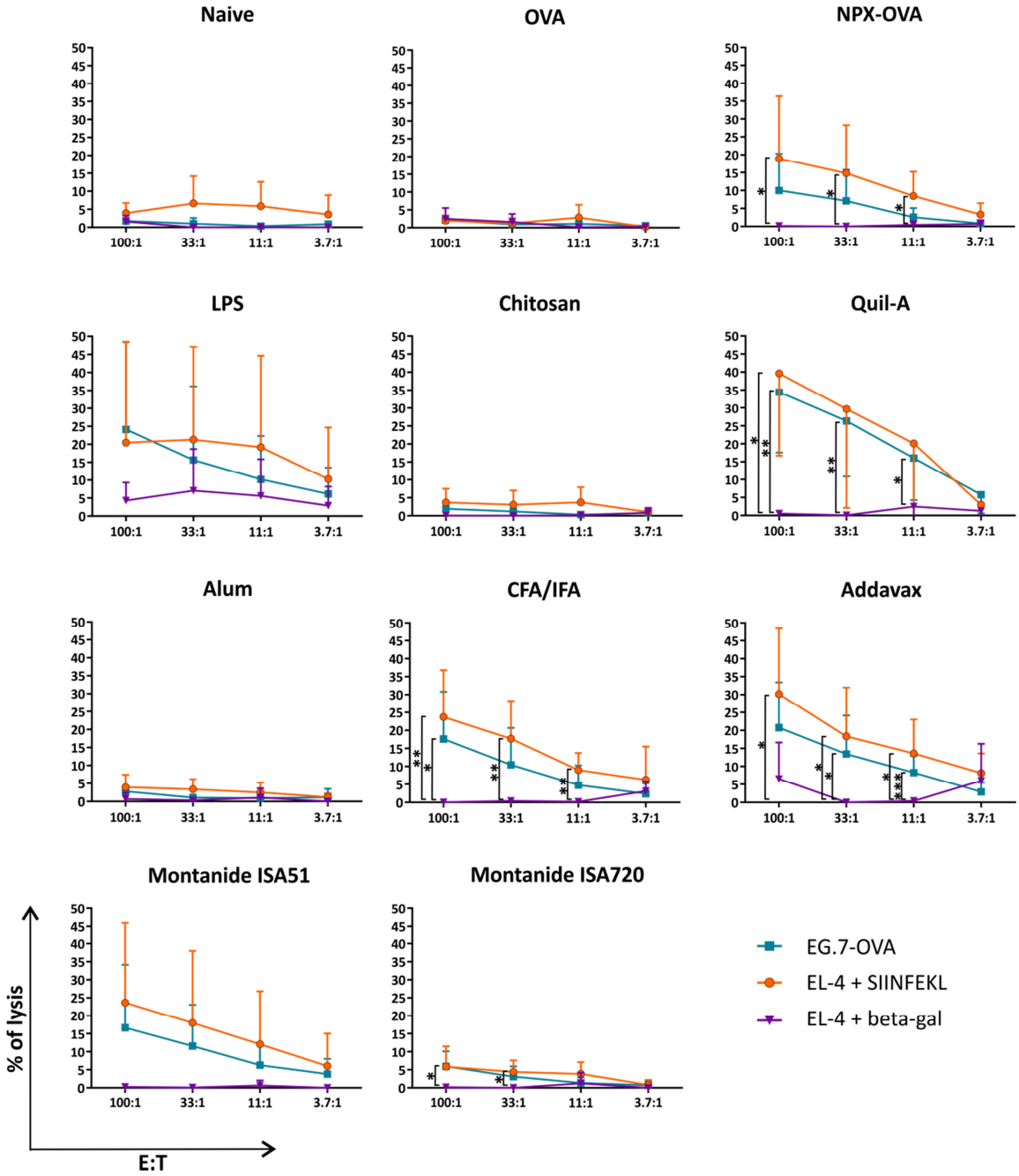

Figure 22. Cytotoxic T cell responses of C57BL/6 mice vaccinated with OVA adjuvanted with different adjuvants. Spleen cells of C57BL/6 mice vaccinated with 10 µg of OVA adjuvanted with different adjuvants (standard schedule), were restimulated *in vitro* at day 30 with EG.7-OVA cells, and evaluated 5 days later for their specific lytic activity against target cell lines by ^{51}Cr release assay. Each graph displays a group of mice tested against three different target cells. Percentages of lysis at different E:T ratio are represented (T: target cells; E: effector cells). Each symbol represents mean values ± SD (n=9 mice/group). Data were analyzed by multiple t-test [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

Data from *in vitro* cytotoxic assays were supported by results of IFN-γ secretion; indeed, ELISA assays performed on supernatants from splenocytes stimulated 72 h in MLTC, showed induction of detectable levels of IFN-γ by NPX-OVA, LPS, Quil-A, CFA/IFA, Addavax and Montanide groups (**Figure 23**).

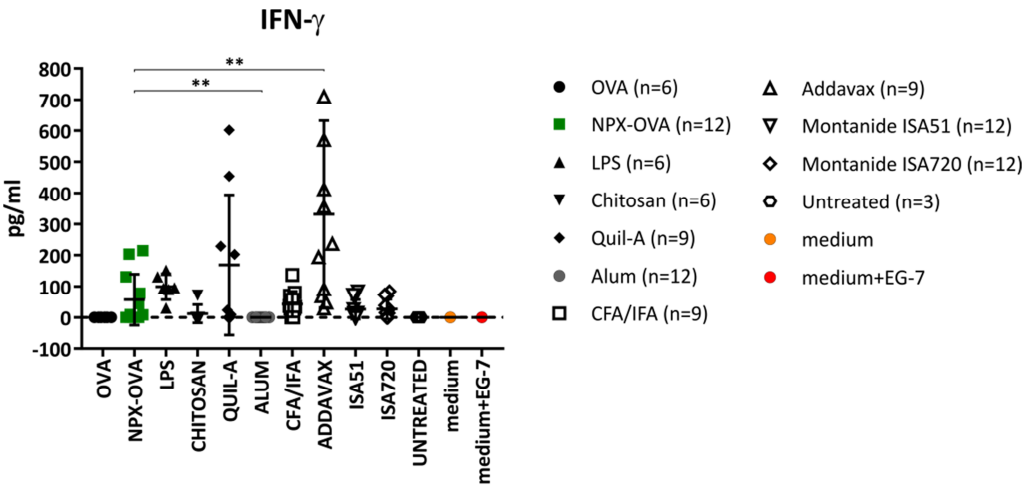

Figure 23. IFN-γ production by splenocytes collected from C57BL/6 mice vaccinated with 10 µg of OVA adjuvanted with different adjuvants. Supernatants from 72 h-stimulated MLTC set up with splenocytes collected at day 30 from vaccinated C57BL/6 mice immunized with 10 µg of OVA adjuvanted with different adjuvants (standard schedule), were analyzed for their cytokine content by ELISA using mouse IFN-γ microplate kit. Multiple t-test was used to compare NPX-OVA with other adjuvants [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

4. Elucidating the mechanism of action of NPX

4.1 Histological assessment of NPX biocompatibility

To assess the safety profile of NPX and evaluate the potential induction of local toxicity in comparison to other commercial adjuvants, a biotolerability analysis was performed. Adjuvants were administered to BALB/c mice through the i.m. route with 10 µg OVA, and both muscle integrity and the presence of local inflammatory reactions were evaluated through histological analysis of tibialis anterior muscles collected at different time points. **Figure 24** shows representative pictures of H&E staining of muscle samples collected 24 hours and 7 days after injection. Intriguingly, NPX-injected muscles disclosed a totally preserved and intact tissue texture, without any trace of inflammatory cell infiltration. Histologies derived from mice immunized with NPX-OVA were indeed comparable with those obtained following injection of OVA alone, which also lacked signs of inflammatory reactions at the injection site. Differently, muscles form mice injected with all the other commercial adjuvants showed clear signs of inflammation, displaying massive recruitment of inflammatory cells at injection site already 24 h after immunization, which still persisted 7 days after injection. In addition, traces of local damage likely caused by the accumulation of oil particles, were detected in specimens from mice immunized with oil-in-water emulsion adjuvants such as CFA/IFA and Montanide.

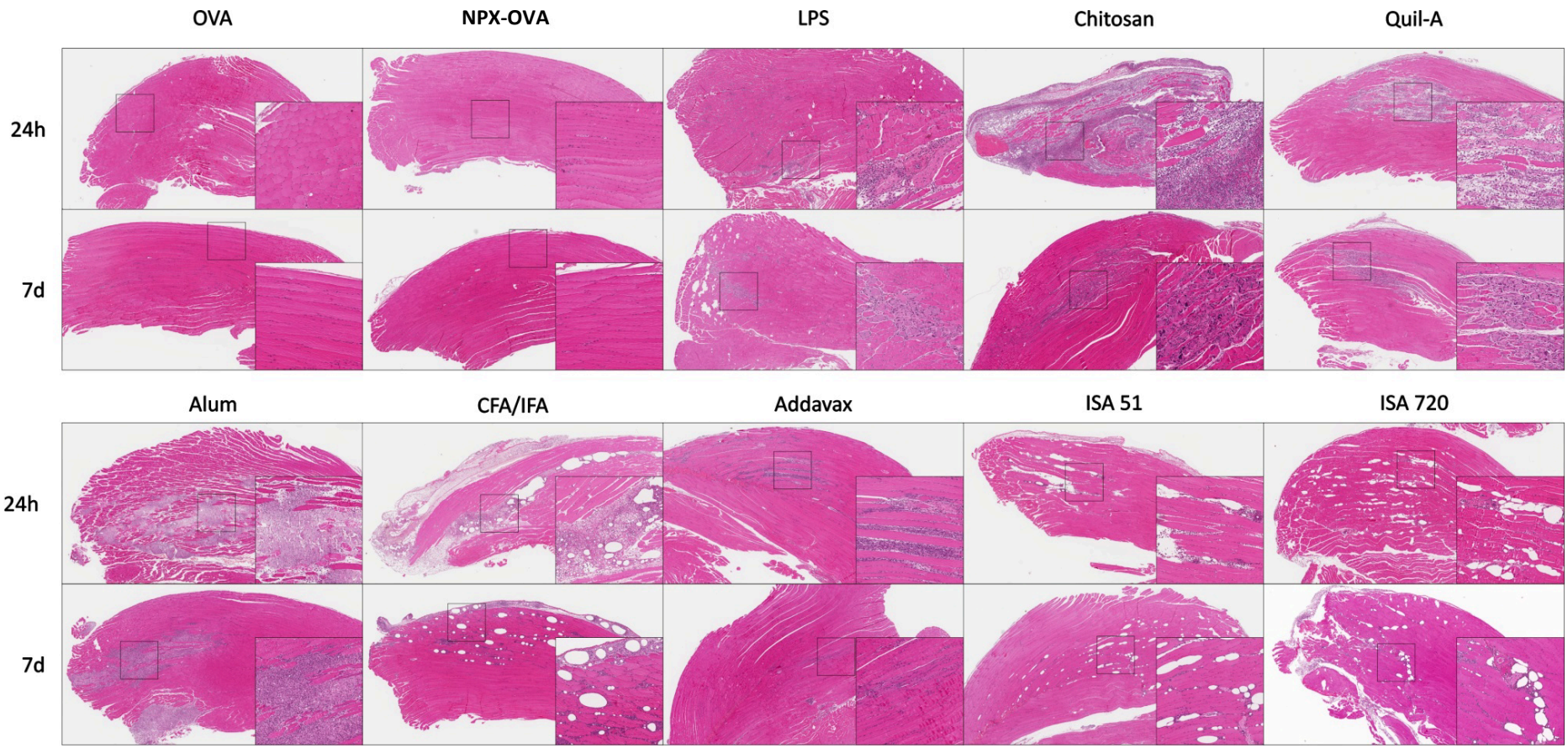

Figure 24. Histological analysis of tibialis anterior muscles from vaccinated BALB/c mice. Representative digital photographs of H&E staining of tibialis muscle collected 24 hours or 7 days after injection of 10 µg OVA and different immunological adjuvants. Histological analyses are reported at 2X magnification, while the inserts show a more detailed view of framed areas (10X).

4.2 Evaluation and characterization of inflammatory cells recruited at the injection site

To confirm the absence of local inflammatory reactions at the site of injection, cell populations recruited into the muscle following injection with NPX were counted and characterized by flow cytometry at different time points. Muscle samples from mice injected with OVA alone, emulsified with Alum, or conjugated to NPX were compared with those from naive non injected mice. At 6h, 24h, and 48h post injection, muscles from each mouse were collected and single cell suspensions were obtained by enzymatic digestion. Following Alum+OVA injection, the recruitment of inflammatory cells at the injection site was already evident by simple enumeration of total cell number, since cell counts registered for Alum-injected muscles were higher than those detected in other groups, and increased over time. Differently, cells enumerated in muscles of NPX-immunized mice were exactly comparable to numbers obtained from naive or OVA-injected muscles, thus confirming the absence of inflammatory cell recruitment after immunization with NPX-OVA (**Figure 25**).

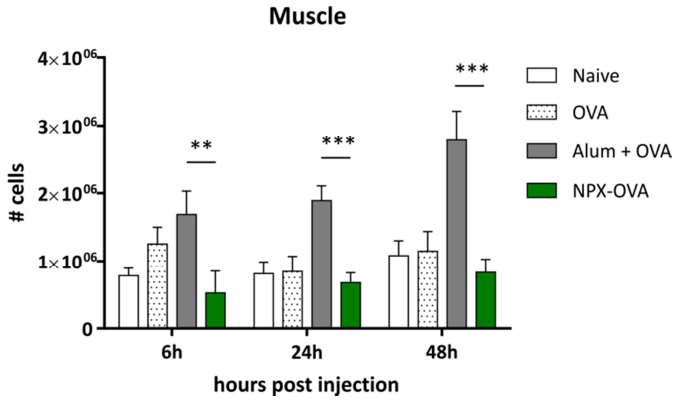

Figure 25. Total numbers of cells counted in tibialis anterior muscles collected 6h, 24h and 48h after injection. Mice were injected in both tibialis anterioris muscles, which were subsequently collected at different time points, digested and evaluated for cell content. Graph reports the number of cells counted in two pooled muscles from each mouse. Bars represent mean values with SD (n=3 in naive, OVA and Alum+OVA groups; n=5 in NPX-OVA group). Data were analyzed using multiple t-test; only statistics for Alum-injected vs. NPX-OVA group are reported [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

For a characterization of the cell infiltrate, single cell suspensions were stained with different inflammatory cell markers in order to evaluate the composition of immune cell populations recruited into the muscle. In particular, multicolor cytometry analysis allowed us to compare the number of DCs ($CD11c^+$, $MHC\text{-}II^+$), macrophages ($CD11b^+$, $F4/80^{high}$), neutrophils ($CD11b^+$, $Ly6G^{high}$), and eosinophils ($CD11b^+$, $Ly6G^{int}$, $F4/80^{int}$, SSC^{high}) between different groups at different time points (**Figure 26**). Results showed a general increase in all the analyzed cell population in the alum-injected group. In particular, the number of DC in muscle showed an increase 48h after

vaccine injection in Alum and OVA groups; the same trend was observed for macrophages, which increased after 24h and 48h both in Alum- and OVA-injected groups. A little but not significant increase of macrophages number was registered 48h post injection also in the NPX-OVA group when compared to naive group. A strong recruitment of neutrophils was evident in the Alum-injected group already after 24h and still after 48h. Differently, the other groups displayed no significant variation in neutrophils number during the monitored period. Also the number of eosinophils gradually increased in the Alum-immunized group, being already significantly higher than that detected in NPX-OVA group at 24h and reaching a peak 48h after injection. Thus, immunization with NPX-OVA induce a null effect on inflammatory cell recruitment, which is comparable to that registered in naive group. On the contrary, both injection of OVA and OVA emulsified with Alum showed to attract different inflammatory cell populations at the site of administration.

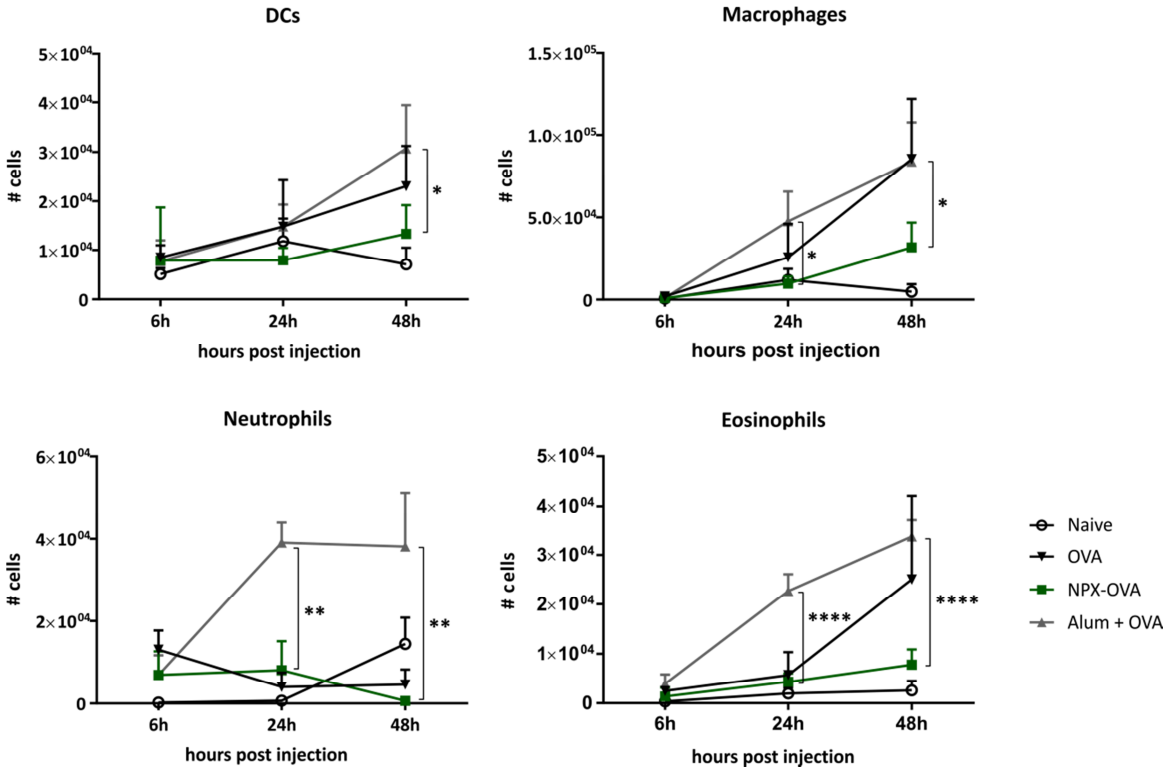

Figure 26. Kinetics of inflammatory cell recruitment into muscle in response to vaccine injection. Mice were injected into two legs, and cell composition of muscles lysates was assessed 6h, 24h and 48h post injection by cytometry. Values show the means + SD of identified cells (n=3 mice in naive, OVA and Alum+OVA groups; n=5 mice in NPX-OVA group). Data were analyzed using multiple t-test; only statistics for Alum-injected *vs.* NPX-OVA group are reported [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

As an additional proof that NPX injection causes only a marginal inflammatory reaction at the injection site, supernatants obtained from muscle tissue digestion were tested for the presence of inflammatory cytokines (IL-10, IL-12p70, IL-1β, IL-6, IL-5, KC/GRO, MCP-1, MIP-1α, and TNFα, **Figure 27**). At an early time point (6h), results showed that all the injected group displayed quite similar amounts of the inflammatory cytokines tested. Differently from muscles injected with OVA and NPX-OVA, which generally displayed constant or decreased quantities of cytokines during the observation time, very high levels of the inflammatory cytokines persisted in supernatants from Alum-injected muscles, remaining detectable also 48h after injection. Illustrative of this trend is the behavior of the monocyte chemoattractant protein-1 (MCP-1), a key regulator of tissue infiltration by monocytes. MCP-1 was indeed detected at high levels in all the groups 6h after injection, but remained significantly higher after 24h and 48h only in the Alum-injected group. As expected and in agreement with the analysis of recruited cells, most of the cytokines detected at high quantities in Alum-injected muscles; also the macrophage inflammatory protein 1 alpha (MIP-1α), IL-6, IL-1β and significantly high amounts of classical pro-inflammatory cytokines like IL-12p70 and TNFα, were detected following injection with Alum. Differently, extremely lower levels of inflammatory cytokines were detected in the group immunized with NPX-OVA, thus confirming the absence of a relevant inflammatory reaction in the muscles of these mice. This observation was supported by the fact that the amounts of cytokines detected in this group were comparable to those found in mice vaccinated with OVA alone and also in non-injected mice. Thus, results obtained from this cytokines analysis in muscles were in agreement with data obtained by cell recruitment analysis, overall indicating that NPX is extremely safe and well tolerated when injected i.m., and appears to act differently from classical immunological adjuvants that trigger strong inflammatory responses at the site of injection.

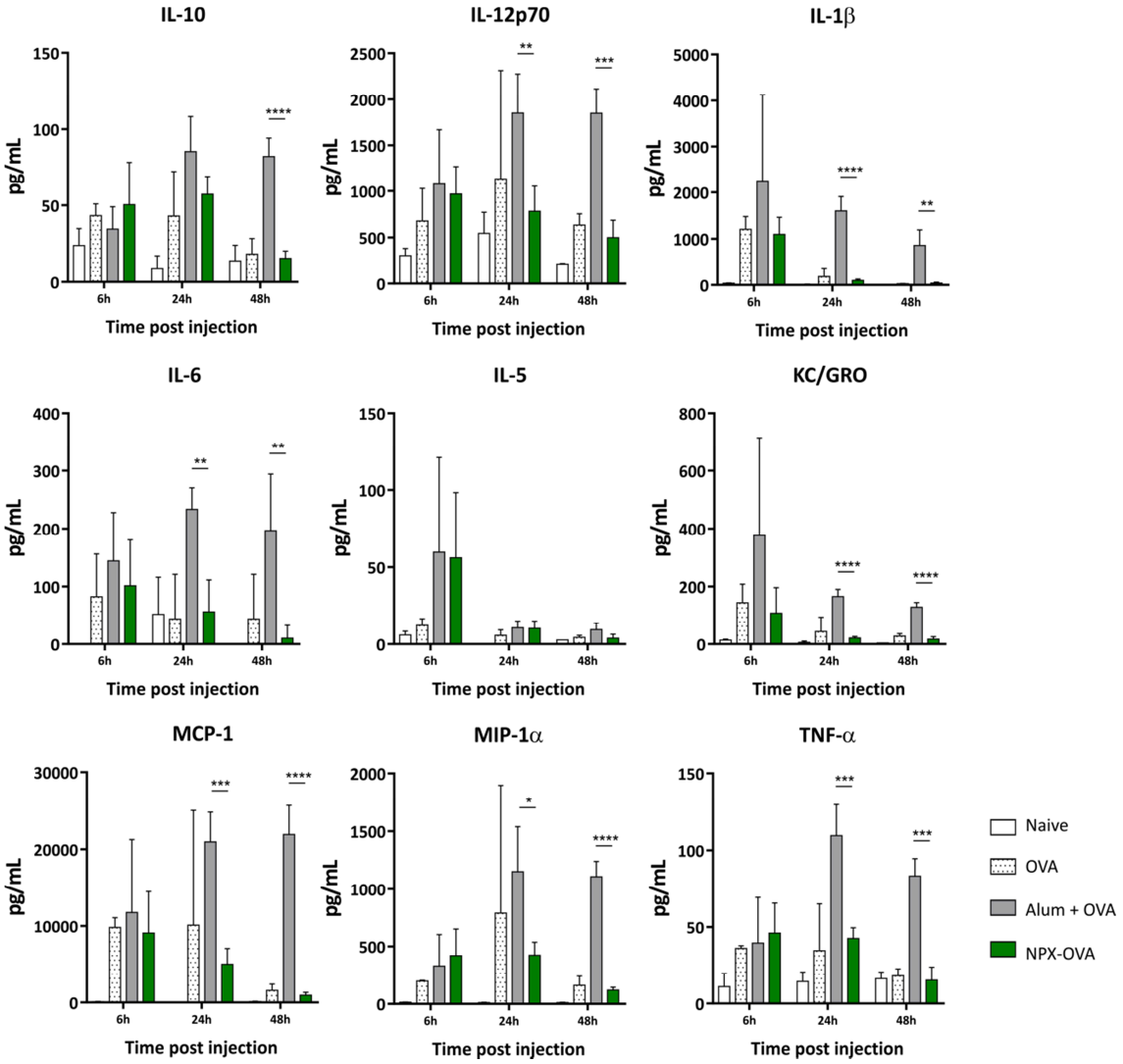

Figure 27. Cytokines content in the muscles in response to vaccine injection. Mice were injected with the indicated compounds and cytokine content in muscle lysates was assessed 6h, 24h and 48h post injection by MSD technology. Bars represent mean + SD (n=3 in naive, OVA and Alum+OVA groups; n=5 in NPX-OVA group). Data were analyzed by multiple t-test; only statistics for Alum-injected *vs.* NPX-OVA group are reported [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

4.3 Assessment of NPX-OVA biodistribution *in vivo*

Previous results indicated that NPX may not act at the injection site to stimulate antigen presenting cells, but might function as carrier for antigen helping its drainage out of injection site. To understand the biological fate of NPX-linked antigens, we performed *in vivo* biodistribution studies aimed at tracking NPX-OVA following injection. To this aim, the antigen was labeled with the Cy5.5 fluorophore and injected in BALB/c mice i.m. either alone, conjugated to NPX, emulsified with Alum or simply mixed with NPX. Since the linking between antigen and NPX is based on covalent bonds and therefore stable, we assumed that the fluorescence emitted by OVA-Cy5.5 conjugated with NPX co-localizes with the presence of NPX. *In vivo* optical imaging of injected mice scanned at different time points allowed us to detect the fluorescence signal, and consequently antigen biodistribution. Results of repeated experiments are expressed in **Figure 28A** as percentages of fluorescence detected either in muscle and injection site-draining lymph node at 4h, 8h, 24h, 48h, and 72h post-injection. Data obtained from this analysis indicated that NPX conjugation to OVA favored retention of the antigen in muscle, disclosing a trend comparable to Alum, that is known to act through a mechanism of depot at the injection site. Differently, OVA injected alone or simply mixed with NPX appeared to be rapidly cleared out from injection site already after 4 hours; fluorescence signals concentrated in liver and bladder, likely as a consequence of protein degradation and dye elimination through kidneys and urine (**Figure 28B**). Interestingly, mice injected with NPX-OVA also showed a clearly detectable fluorescent signal that promptly localized at the inguinal lymph node (highlighted by red circles); this signal was significantly higher than that registered in all the other groups at early time points (4h and 8h), indicating that NPX fosters antigen accumulation in the draining lymph node. This behavior likely relies on the presence of NPX-receptors expressed by the endothelium of lymphatic vessels, which can help and accelerate the drainage of NPX-OVA trough the lymphatic system to the lymph node, where it may then act by stimulating DCs.

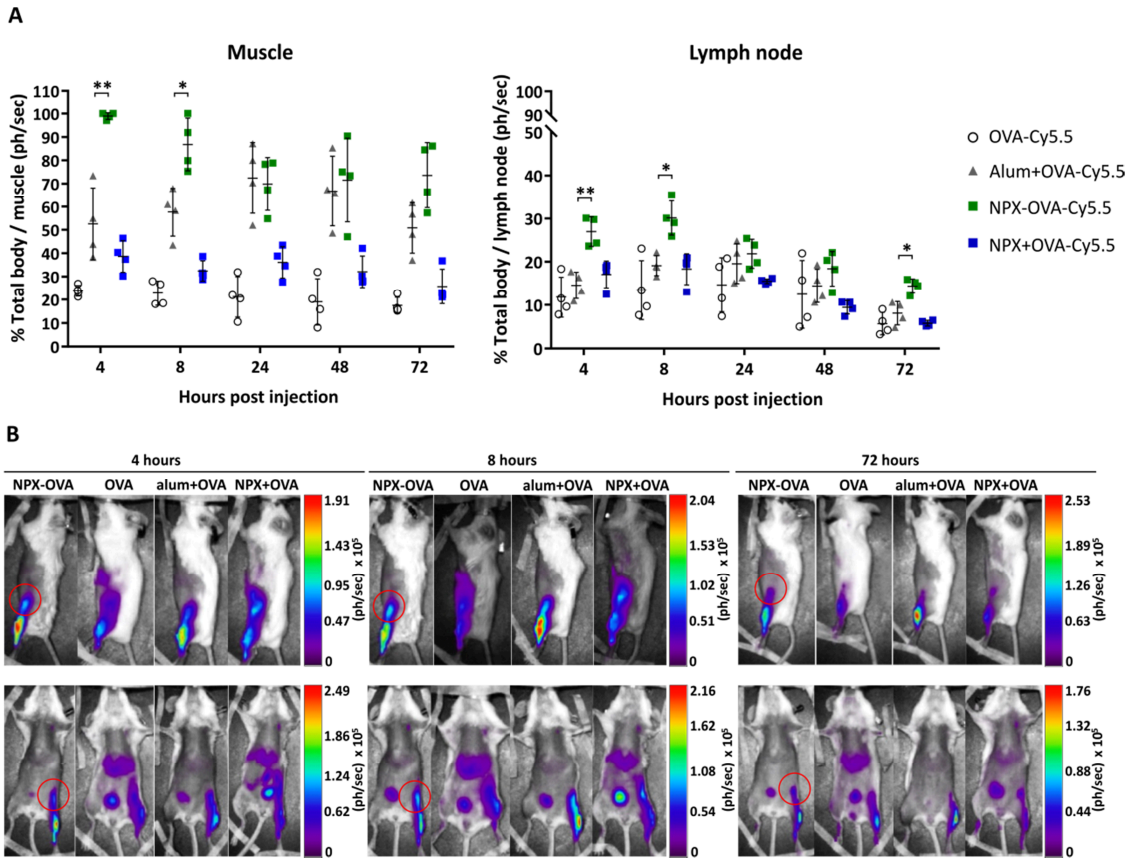

Figure 28. *In vivo* biodistribution of OVA-Cy5.5 in muscle and lymph node at 4, 8, 24, 48 and 72 hours post injection. A) Percentage of accumulated ph/sec in muscle and lymph node at different time points. Data were analyzed using multiple t-test; only statistics for Alum-injected *vs.* NPX-OVA group are reported [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated]. **B)** Representative pictures of live animals taken at 4, 8 and 72 hours post injection. One representative animal/group is depicted during lateral (top panel) or frontal (bottom panel) scans at different time points.

The preferential accumulation of fluorescent NPX-conjugated OVA in lymph node following i.m. injection was also demonstrated by fluorescence microscopy of lymph node specimens (**Figure 29**). To finely display the biodistribution of OVA-Cy5.5, draining inguinal lymph nodes were isolated 6 hours post-injection, sections were stained with DAPI and images were captured at different magnifications. As reported in **Figure 29A**, the presence of the Cy5.5 fluorophore in the lymph node was visually and macroscopically detectable in specimens from NPX-injected mice but not in lymph nodes from Alum-injected mice. When analyzed by fluorescence microscopy, only NPX but not Alum specimens showed a specific fluorescent signal, which at higher magnification (**Figure 29B** and **Figure 29C**) appeared to be localized and concentrated in the cytoplasm of few cells spread in the tissue. While the specific identity of these cells remain to be determined, we speculate that they could be APCs, in particular DCs, that engulf the antigen in the lymph node.

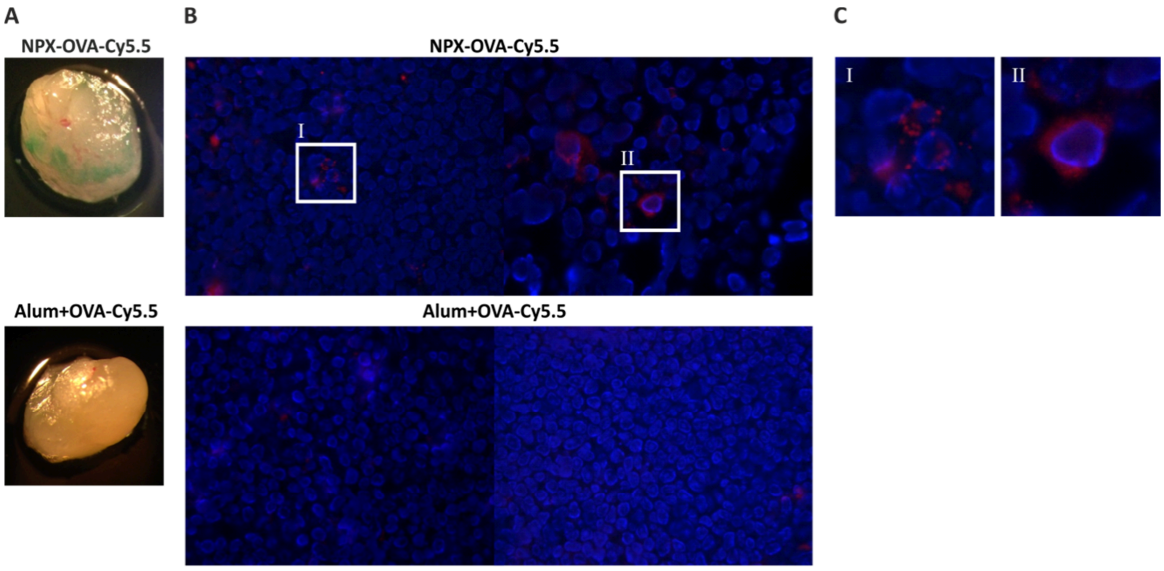

Figure 29. Localization of OVA-Cy5.5 in lymph node specimens taken from Alum- or NPX-immunized mice 6h post injection. A) Pictures of inguinal lymph nodes collected from Alum- or NPX-injected mice 6 hours post injection (Leica Wild M3B Stereo Microscope, 6.4X magnification). **B)** Representative fluorescence microscopy images of lymph nodes specimens from mice immunized with Alum + OVA-Cy5.5 or NPX-OVA-Cy5.5. Specimens were stained with DAPI (blue) and OVA-Cy5.5 fluorescence (red) was detected at 10X magnification. **C)** Detailed view of Cy5.5-specific signals in areas framed by the white squares from B (Oil 60X magnification).

4.4. Studies on the effects exerted by NPX-OVA on DCs

The role of DCs in NPX adjuvanticity is supported by wide evidences in literature indicating that low molecular weight (LMW) derivatives of NPX are able to act as DAMPs, stimulating DCs though interaction with specific TLRs. LMW-NPX are physiologically generated by the specific enzyme NPXase, which is widely expressed in different tissues and has a central role in NPX catabolism. Thus, our hypothesis is that NPX-OVA, once injected, undergoes a degradation process leading to the production of LMW derivatives that are rapidly captured by the lymphatic vessels helped by the presence of NPX receptors. LMW products of NPX degradation encounter DCs in the lymph node and induce their stimulation trough binding of TLRs, consequently increasing their capacity to engulf and present the antigen bound to NPX.

In an attempt to demonstrate the hypothesis of the involvement of NPX degradation as a fundamental step of its mechanism of action as adjuvant, the capability of the NPXase enzyme to generate LMW fragments from NPX-OVA bioconjugate was tested *in vitro*. Firstly, the molecular weight of fragments generated by digestion of unconjugated 200 kDa NPX with NPXase, was calculated using a standard curve obtained by HPLC analysis of different sized NPX fragments of known concentration (**Figure 30A**). Results from this quantitative analysis indicated that NPXase was able to digest *in vitro* 200 kDa NPX, generating LMW NPX derivatives of a size ranging from

0.5 to 129 kDa. Then, the same digestion protocol was used to test NPX-OVA digestion. Repeated experiments showed that incubation of NPX-OVA with NPXase was efficient in generating multiple products of variable sizes (**Figure 30B**). Although this analysis was not quantitative, since it was not possible to determine the size of fragments due to the presence of the protein OVA bound to NPX, this assays clearly demonstrated the generation of different and small sized-products from *in vitro* digestion of NPX-OVA with NPXase enzyme.

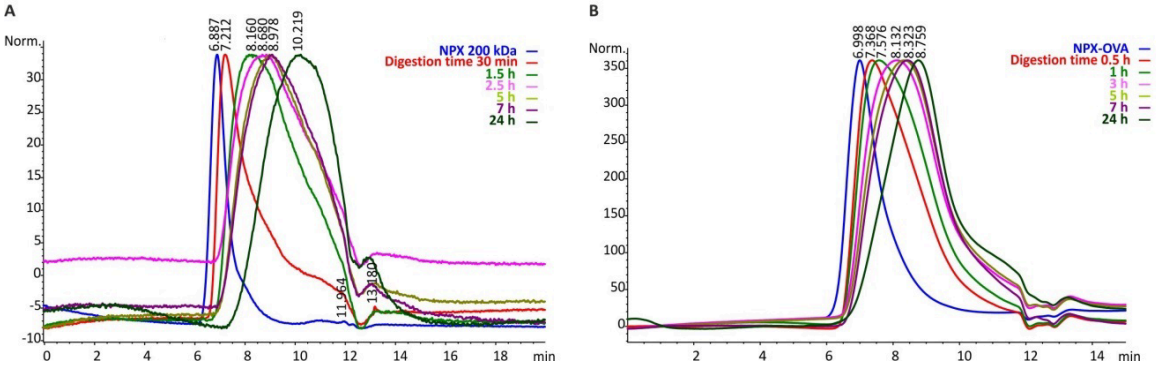

Figure 30. HPLC analysis of digestion kinetics of 200 kDa NPX (A) and NPX-OVA (B) with NPXase.

Once demonstrated that NPX-OVA can be digested *in vitro* by the NPXase enzyme, next we wondered whether LMW NPX derivatives could stimulate DCs maturation *in vitro*. To this end, we measured the induction of maturation markers in BMDCs cultures stimulated with commercial LMW fragments of NPX (**Figure 31**). Results from repeated experiments disclosed that stimulation with small-sized NPX derivatives increased the expression of maturation markers on the surface of BMDCs, at a level similar or even significantly higher than LPS that was used as positive control of DCs maturation, thus confirming the ability of LMW NPX to act as DAMPs.

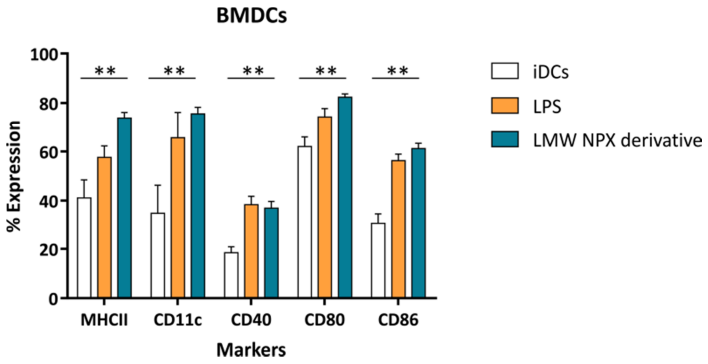

Figure 31. Percentage of expression of DCs maturation markers on the surface of BMDCs. BMDCs cultures were stimulated from day 0 with LMW NPX or from day 6 with LPS and cytofluorimetric detection of MHC-II, CD40, CD80, CD86 markers was performed at day 9 after stimulation. Stimulation with LPS was used as control of DCs maturation while un-stimulated iDCs were collected and stained at day 6 as negative control. Bars represent mean of 3 separate experiments + SD. Data were analyzed by multiple t-test; only statistics for iDCs group *vs.* LMW NPX-stimulated groups are reported [*=P<0.05, **= P<0.01, ***= P<0.001, ****= P<0.0001, not statistically significant (p<0.05) if not indicated].

Further experiments aimed at comparing the effect of stimulation of BMDCs with NPX-OVA, NPX-OVA digested with NPXase and commercial LMW NPX derivative are currently ongoing. Data obtained in a preliminary experiment (**Figure 32**) suggest that both LMW NPX derivatives and NPX-OVA digested with NPXase were able to induce an increased expression of CD80 and CD86 maturation markers, at a level comparable to that obtained with LPS. Surprisingly, also NPX-OVA seemed to induce an increase of those markers even if at lower extent when compared to digested NPX-OVA or commercial LMW NPX fragments. We speculate that NPX-OVA might be digested *in vitro* due to the action of NPXase expressed by BMDCs, which in turn would autocrinally stimulate themselves to mature and activate; Nonetheless, additional assays are required to confirm this preliminary result.

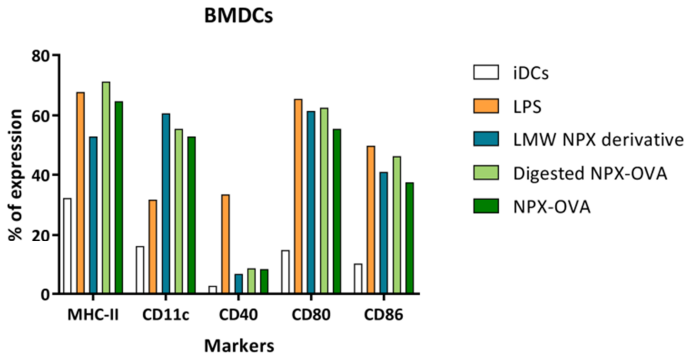

Figure 32. Percentage of expression of DCs maturation markers on the surface of BMDCs. BMDCs were stimulated with LPS, LMW NPX derivative, NPX-OVA or NPX-OVA digested with NPXase, as previously described and stained with MHC-II, CD11c, CD40, CD80 and CD86 markers. Data are representative of a single experiment.

Discussion

This project aimed at exploring the potentiality of NPX as an effective and safe adjuvant for protein-based vaccination studies, by assessing its biological behaviour in addressing the main requirements that a putative ideal adjuvant should possess. Therefore, NPX ability of stimulating humoral and cytotoxic immune responses was tested *in vivo,* together with its biotolerability and safety profile. Moreover, additional work was carried out to elucidate the mechanism(s) of action of NPX.

First, our results clearly establish that NPX leads to the elicitation of strong Ab responses against the OVA model antigen, and support the neat superiority of NPX over Alum. NPX was indeed efficient in inducing OVA-specific IgG production also after a single injection or immunization with very low antigen doses, thus allowing boosting and antigen sparing, two of the most wanted features for an ideal adjuvant. Conversely, Alum required more injections and higher antigen concentration to stimulate detectable OVA-specific IgG, and proved not to be endowed with the same remarkable properties. NPX-induced humoral response was showed to be strong and long-lasting, indicating that NPX-adjuvanted immunization generates durable Ab responses and can likely confer long-term protection. We also succeeded in demonstrating that NPX adjuvant effect is clearly related to its chemical conjugation to the protein antigen, as the simple mixing of NPX to OVA did not induce a humoral response against the model antigen. Since this prerequisite for NPX adjuvanticity might represent a limitation for an industrial scale-up and a reproducible manufacturing of NPX-based vaccines, pilot experiments are planned to address this conjugation issue by investigating whether NPX derivatives capable of physically entrapping the protein antigen still maintain the adjuvant effect.

To strength the concept that NPX can be regarded as a new powerful adjuvant prototype, we also performed a large comparative study where NPX was tested in parallel with other commercial adjuvants (Quil-A, Addavax, Complete/Incomplete Freund's Adjuvant, Chitosan, LPS, and Montanide). Results of such studies disclosed that the IgG production induced by NPX was comparable or even superior to that elicited by all the other adjuvants tested. Only the humoral response stimulated by CFA/IFA was higher than that generated with NPX-OVA, but it is worth to note that NPX was always strikingly more efficient than those adjuvants already approved for clinical use (e.g. Alum, Addavax). Such competitive profile of NPX against other commercial adjuvants was evident in repeated experiments performed with different immunization conditions, and in two different mouse strains. Notably, with this large comparative study we provided a proof of the importance of comparing different adjuvants using the same model

antigen, in order to establish their behavior and efficacy under different immunization conditions.

Another crucial point in favour of the NPX adjuvanticity is that NPX-induced humoral response, differently from that elicited by Alum, was also characterized by the production of IgG_{2a} and IgG_{2b} subclasses, which are normally linked to a Th1-skewed immune response. The production of IgG_1, IgG_{2a} and IgG_{2b} Abs observed in NPX-vaccinated mice suggests that NPX likely elicits both Th1 and Th2 responses, thus promoting a more balanced reaction in comparison to Alum, which is known to generate a strong Th2-skewed response. Thus far, most of the commonly used adjuvants in vaccines approved for human use, have failed in stimulating appropriate cytotoxic T cell responses; indeed, a major challenge in the field of adjuvant development is to find a compound able to elicit CMI. Thus, a crucial point for a complete characterisation of NPX was the assessment of its ability to induce also a functional Th1 immune response. Experiments aimed at clarifying this aspect showed controversial results: although in our hands immunization of mice with NPX-OVA led to the induction of CTL and IFN-γ production in MLTC that were comparable to those stimulated by well-known Th1-inducing adjuvants, no relevant numbers of SIINFEKL-specific $CD8^+$ cells were detected by pentamer staining in different compartments following NPX-OVA immunization. In this regard, the fact that pentamer staining experiments were performed in a host laboratory (Statens Serum Institute, Copenhagen, Denmark) under slightly different experimental conditions, must be taken into account during data interpretation. For example, the concentration of OVA used for mice immunization in such experiments was 1 μg instead of the standard dose of 10 μg, and this different antigenic load may have influenced the induction of antigen-specific cytotoxic cells. Thus, the dissection of this point and the assessment of the effective absence/presence of SIINFEKL-specific $CD8^+$ T cells need to be confirmed by repeating the experiments with a higher antigen dose. Although the question whether NPX is able to promote a CTL response remains still open, the data collected until now are strongly suggesting that NPX adjuvanticity prevalently resides on its ability to efficiently enhance the magnitude, breadth, quality, and longevity of specific humoral responses to antigens.

In this work, another remarkable feature of NPX as an adjuvant was highlighted: its biocompatibility at the injection site and its generally safe profile; indeed, no systemic neither local side effects occurred in vaccinated mice. As emerged from H&E staining of injected muscles, NPX did not trigger any apparent inflammatory reaction at the site of injection and preserved muscle integrity, differently from all the other adjuvants tested. Results obtained by

cell recruitment and cytokines analysis of muscle lysates, overall confirmed H&E data pointing out that NPX is extremely safe and well tolerated when injected i.m., apparently acting differently from classical immunological adjuvants that trigger strong inflammatory responses at the site of inoculation.

Paradoxically, the mechanism of action of the most widely used adjuvant in history, namely Alum, has been elucidated only recently, after several decades of extensive use and billions of administrations *(Wen Y and Shi Y, 2016)*. In an attempt of providing a comprehensive view of NPX, we also carried out a number of studies aimed at investigating its mechanism of action. Results of NPX-OVA biodistribution analysis after i.m. injection disclosed that antigen-associated fluorescent signal at the site of inoculation was comparable between NPX-OVA and Alum+OVA groups, along the monitored period. This suggests that NPX-OVA, similarly to Alum, may act as a depot by retaining the antigen at the administration site. Nonetheless, this hypothesis is apparently in contrast with the classical concept of "danger" signal, as evidences obtained by both H&E staining and cytometry analysis of digested muscle tissue showed the absence of inflammatory cells *in situ*. A potential explanation could be that NPX-OVA, being fully biocompatible, is per se immunologically inert in its native form at the site of inoculation, and hence does not recruit and activates inflammatory cells and APCs. Therefore, the observed depot effect is likely an epiphenomenon not directly involved in the mechanism of action through which NPX exerts its adjuvant function. This hypothesis is consistent with the detection of an increased and clearly defined antigen-associated fluorescence in the lymph node draining the injection site, at least at early time points. Indeed, the fluorescent signal detected in draining lymph nodes of NPX-OVA injected mice appeared to be significantly higher at 4 and 6 hours after administration than the signal detected in the other groups, indicating that NPX favors the rapid localization of the antigen to the loco-regional lymph nodes. These results, which found a confirmation in the fluorescence microscopy evaluation of lymph node sections, suggest that NPX might act by promoting the rapid migration and accumulation of the antigen to the draining lymph node, where the encounter with APCs and responding lymphoid cells is facilitated.

The fact that NPX adjuvant effect relies on the chemical conjugation to the antigen and that this latter is preferentially drained to the afferent lymph node when conjugated to NPX, is not casual. The rapid drainage of the antigen to the lymph node mediated by NPX can indeed be explained by the presence of a NPX-specific receptor, which has been reported to be expressed by endothelial cells of lymphatic vessels. The affinity of the NPX receptor for NPX-OVA could actually favor the bioconjugate to enter the lymphatic vessels, thus facilitating the migration and the drainage of NPX-OVA to the lymph node, where it is preferentially accumulated. At the same

time, we speculate that NPX may undergo degradation by NPXase enzymes present in tissues. Indeed, LMW NPX products generated from NPX digestion may function as DAMPs activating DCs trough binding specific TLRs and thus stimulating antigen recognition and presentation to effector cells. This potential scenario was assessed by digestion experiments of NPX-OVA with NPXase enzyme, where results confirmed the production of fragments of NPX at different molecular size. In parallel, BMDCs stimulation experiments confirmed that LMW NPX derivatives induce DCs maturation *in vitro.*

Taken together, these results represent important advancements in the dissection of NPX mechanism of action, but also highlight the complexity of the adjuvants world, and indicate that the way is still long to fully understand the exact mechanism/s and pathway/s through which NPX exerts its adjuvant activity. Further work is indeed required to determine whether products of NPX-OVA digestion *in vitro* are effectively capable of directly stimulating DCs, thus confirming the ability of NPX-OVA digestion products to act as TLRs agonists and thus supporting our hypotheses about NPX action in the lymph node. Additionally, *in vivo* studies are currently ongoing with the aim of i) tracking NPX-OVA and assessing whether it has the ability to induce DCs maturation in the draining lymph node, ii) verifying the activation status of DCs subsets and potentially the presence *in vivo* of other APCs involved in the process.

Finally, one of the major challenge for an ideal adjuvant remains to be investigated: its effectiveness not only with surrogate antigens but also in proper experimental models of disease. Regarding this point, very promising results have been already obtained in our laboratory by studying the application of NPX chemically conjugated to the rat form of Her2/neu oncoantigen, as an anticancer vaccine formulation in breast cancer models. Unpublished data showed the successful application of anti-Her2/neu NPX-adjuvanted vaccination. Indeed, a NPX-Her2/neu bioconjugate proved to be highly effective in both prophylactic and therapeutic settings, eliciting strong immune responses able to prevent or significantly delay tumor growth in syngeneic and spontaneous breast cancer mouse models. Ongoing experiments are intended to validate the efficiency of NPX adjuvant in an infectious disease setting; in particular, the protective efficacy of the immune response elicited by immunization with NPX conjugated with a rabies virus antigen is being investigated and tested in challenge experiments in a rabies infections model of disease.

In summary, this work advances NPX as a new and promising candidate adjuvant, providing insights into its major activity and mechanistic features, together with assessment of biotolerability. Although NPX ability to produce cell-mediated immunity remains to be clarified, the potency of humoral responses induced, which outperformed results obtained with other

clinical-grade adjuvants, sets an important proof that NPX can be regarded as an excellent alternative for the design of safe and efficient protein-based vaccine formulations.

Abbreviations

A

Ab: antibody

ADCC: antibody-dependent cell cytotoxicity

Ag: antigen

AIM2: absence in melanoma 2-like receptor

Akt: protein kinase B

APC: antigen-presenting cells

AS04: adjuvant system 04

ASC protein: apoptosis-related speck-like protein containing a CARD domain

C

CAF: Cationic adjuvant formulation

CARD: caspase activation and recruitment domain

CCL-2: chemokine (C-C motif) ligand 2

CCR-2: C-C chemokine receptor type 2

CDC: complement-dependent cytotoxicity

CDC: complement-dependent cytotoxicity

CLRs: C-type lectin receptors

CMI: cell-mediated immunity

CpG: cytosine phosphoguanosine

CTL: cytotoxic $CD8^+$ T lymphocytes

D

DAMPs: damage-associated molecular patterns

DCs: dendritic cells

DDA: Dimethyldioctadecylammonium bromide

dLN: draining lymph node

dsRNA: double-stranded RNA

DT: diphtheria-tetanus

E

ELISA: enzyme-linked immunosorbent assay

E:T: effector:target

F

FBS: fetal bovine serum

FDA: Food and Drug Administration

FDCs: follicular dendritic cells

G

GCs: germinal centers

GM-CSF: granulocyte-macrophage colony-stimulating factor

H

HBsAg: recombinant HBV surface antigen

HBV: hepatitis B virus

HCV: hepatitis C virus

HIV: human Immunodeficiency Virus

HPV: human papillomavirus

HSV: herpes simplex virus

I

i.m.: intramuscular

IFA: incomplete Freund's adjuvant

IFN: interferon

Ig: immunoglobuline

IHC: immunohistochemistry

IL: interleukin

ISCOMs: immunostimulatory complexes

L

LN: lymph node

LPS: lipopolysaccharide

M

mAbs: monoclonal antibodies

MAPK: mitogen-activated protein kinase

MHC: major histocompatibility complex

MPL: 3-O-desacyl-42-monophosphoryl lipid A

MW: molecular weight

N

NF-κB: nuclear factor-κB

NK: natural killer

NLRP3: pyrin-domain-containing 3 inflammasome

NLRs: NOD-like receptors

NPs: natural polymers

NSCLC: non-small-cell lung cancer

O

O/W: oil-in-water

ODN: oligodeoxynucleotides

OVA: ovalbumin

P

PAMPs: pathogen-associated molecular patterns

PBS: phosphate buffered saline

Poly(I:C): polyiosinic:polycytidylic acid

PRRs: pattern recognition receptors

R

RAG: recombination activating gene

RIG-I: retinoic-inducible gene-1

RLRs : RIG-I-like receptors

RT: room temperature

S

SD: standard deviation

SPF: Specific Pathogen Free

ssRNA: single-stranded RNA

T

TA: tibialis anterior

TCR: T-cell receptor

Tfh: $CD4^+$ T follicular helper lymphocytes

TGF: transforming growth factor

Th: $CD4^+$ T helper lymphocytes

TIR: IL1-R/TLR superfamily

TLRs: Toll-like receptors

TNF: tumor necrosis factor

Tregs: $CD4^+CD25^+FOXP3^+$ regulatory T lymphocytes

Tween 80: polysorbate 80

V

VLPs: virus-like particles

W

W/O: water-in-oil

WHO: World Health Organization

Bibliography

Agger EM, Rosenkrands I, Hansen J, et al. Cationic Liposomes Formulated with Synthetic Mycobacterial Cordfactor (CAF01): A Versatile Adjuvant for Vaccines with Different Immunological Requirements. Rodrigues MM, ed. PLoS ONE. 2008;3(9):e3116. doi:10.1371/journal.pone.0003116.

Allison AG, Gregoriadis G. Liposomes as immunological adjuvants. Nature. 1974 Nov 15;252(5480):252. PubMed PMID: 4424229.

Amidi, M., Mastrobattista, E., Jiskoot, W. & Hennink, W.E. Chitosan-based delivery systems for protein therapeutics and antigens. Adv. Drug Deliv. Rev. 62, 59–82 (2010)

Ammi R, De Waele J, Willemen Y, Van Brussel I, Schrijvers DM, Lion E, Smits EL. Poly(I:C) as cancer vaccine adjuvant: knocking on the door of medical breakthroughs. Pharmacol Ther. 2015 Feb;146:120-31. doi: 10.1016/j.pharmthera.2014.09.010. Epub 2014 Oct 2. Review. PubMed PMID: 25281915.

André FE. Vaccinology: past achievements, present roadblocks and future promises. Vaccine. 2003 Jan 30;21(7-8):593-5. PubMed PMID: 12531323.

Apostólico Jde S, Lunardelli VA, Coirada FC, Boscardin SB, Rosa DS. Adjuvants: Classification, Modus Operandi, and Licensing. J Immunol Res. 2016;2016:1459394. doi: 10.1155/2016/1459394. Epub 2016 May 4. Review. PubMed PMID: 27274998; PubMed Central PMCID: PMC4870346.

Aucouturier J, Dupuis L, Deville S, Ascarateil S, Ganne V. Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. Expert Rev Vaccines. 2002 Jun;1(1):111-8. PubMed PMID: 12908518.

Awate S, Babiuk LA, Mutwiri G. Mechanisms of action of adjuvants. Front Immunol. 2013 May 16;4:114. doi: 10.3389/fimmu.2013.00114. eCollection 2013. PubMed PMID: 23720661; PubMed Central PMCID: PMC3655441.

Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat Rev Immunol. 2010 Nov;10(11):787-96. doi: 10.1038/nri2868. Epub 2010 Oct 15. Review. PubMed PMID: 20948547.

Baxter D. Active and passive immunity, vaccine types, excipients and licensing. Occup Med (Lond). 2007 Dec;57(8):552-6. Review. PubMed PMID: 18045976. André FE. Vaccinology: past achievements, present roadblocks and future promises. Vaccine. 2003 Jan 30;21(7-8):593-5. PubMed PMID: 12531323.

Bergmann-Leitner ES, Leitner WW. Adjuvants in the Driver's Seat: How Magnitude, Type, Fine Specificity and Longevity of Immune Responses Are Driven by Distinct Classes of Immune Potentiators. Vaccines (Basel). 2014 Apr 10;2(2):252-96. doi: 10.3390/vaccines2020252. Review. PubMed PMID: 26344620; PubMed Central PMCID: PMC4494256.

Beverley PC. Immunology of vaccination. Br Med Bull. 2002;62:15-28. Review. PubMed PMID: 12176847.

Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, Maitland M, Norgard MV, Plevy SE, Smale ST, Brennan PJ, Bloom BR, Godowski PJ, Modlin RL. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science. 1999 Jul 30;285(5428):732-6. PubMed PMID: 10426995.

Busch RA, Jonker MA, Pierre JF, Heneghan AF, Kudsk KA. Innate Mucosal Immune System Response of BALB/c vs C57BL/6 Mice to Injury in the Setting of Enteral and Parenteral Feeding. JPEN J Parenter Enteral Nutr. 2016 Feb;40(2):256-63. doi: 10.1177/0148607114558489. Epub 2014 Nov 17. PubMed PMID: 25403938; PubMed Central PMCID: PMC4433866.

Calabro S, Tortoli M, Baudner BC, Pacitto A, Cortese M, O'Hagan DT, De Gregorio E, Seubert A, Wack A. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. Vaccine. 2011 Feb 17;29(9):1812-23. doi: 10.1016/j.vaccine.2010.12.090. Epub 2011 Jan 6. PubMed PMID: 21215831.

Chen GY, Nuñez G. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol. 2010 Dec;10(12):826-37. doi: 10.1038/nri2873. Epub 2010 Nov 19. Review. PubMed PMID: 21088683; PubMed Central PMCID: PMC3114424.

Cheng YS, Xu F. Anticancer function of polyinosinic-polycytidylic acid. Cancer Biol Ther. 2010 Dec 15;10(12):1219-23. Epub 2010 Dec 15. Review. PubMed PMID: 20930504.

Chowdhury RR, Ghosh SK. Phytol-derived novel isoprenoid immunostimulants. Front Immunol. 2012 Mar 22;3:49. doi: 10.3389/fimmu.2012.00049. eCollection 2012.

Coffman RL, Sher A, Seder RA. Vaccine adjuvants: putting innate immunity to work. Immunity. 2010 Oct 29;33(4):492-503. doi: 10.1016/j.immuni.2010.10.002. Review. PubMed PMID: 21029960; PubMed Central PMCID: PMC3420356.

Crombet Ramos T, Rodríguez PC, Neninger Vinageras E, Garcia Verdecia B, Lage Davila A. CIMAvax EGF (EGF-P64K) vaccine for the treatment of non-small-cell lung cancer. Expert Rev

Vaccines. 2015;14(10):1303-11. doi: 10.1586/14760584.2015.1079488. Epub 2015 Aug 20. Review. PubMed PMID: 26295963.

De Gregorio E, Tritto E, Rappuoli R. Alum adjuvanticity: unraveling a century old mystery. Eur J Immunol. 2008 Aug;38(8):2068-71. doi: 10.1002/eji.200838648. PubMed PMID: 18651701.

De Silva NS, Klein U. Dynamics of B cells in germinal centres. Nat Rev Immunol. 2015 Mar;15(3):137-48. doi: 10.1038/nri3804. Epub 2015 Feb 6. Review. PubMed PMID: 25656706; PubMed Central PMCID: PMC4399774.

Desmet CJ, Ishii KJ. Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. Nat Rev Immunol. 2012 Jun 22;12(7):479-91. doi: 10.1038/nri3247. Review. PubMed PMID: 22728526.

Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, Kielland A, Vosters O, Vanderheyde N, Schiavetti F, Larocque D, Van Mechelen M, Garçon N. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol. 2009 Nov 15;183(10):6186-97. doi: 10.4049/jimmunol.0901474. Epub 2009 Oct 28. PubMed PMID: 19864596.

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. PubMed PMID: 11592066.

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. PubMed PMID: 10519932.

Flach TL, Ng G, Hari A, Desrosiers MD, Zhang P, Ward SM, Seamone ME, Vilaysane A, Mucsi AD, Fong Y, Prenner E, Ling CC, Tschopp J, Muruve DA, Amrein MW, Shi Y. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. Nat Med. 2011 Apr;17(4):479-87. doi: 10.1038/nm.2306. Epub 2011 Mar 13. PubMed PMID: 21399646.

Garcia-Fuentes M, Alonso MJ. Chitosan-based drug nanocarriers: where do we stand? J Control Release. 2012 Jul 20;161(2):496-504. doi: 10.1016/j.jconrel.2012.03.017. Epub 2012 Mar 23. Review. PubMed PMID: 22480607.

Garçon N, Vaughn DW, Didierlaurent AM. Development and evaluation of AS03, an Adjuvant System containing α-tocopherol and squalene in an oil-in-water emulsion. Expert Rev

Vaccines. 2012 Mar;11(3):349-66. doi: 10.1586/erv.11.192. Review. PubMed PMID: 22380826.

Glenny A., Pope C., Waddington H. (1926)"The antigenic value of toxoid precipitated by potassium alum" J Pathol Bateriol 29:31-40

Guy B. The perfect mix: recent progress in adjuvant research. Nat Rev Microbiol. 2007 Jul;5(7):505-17. Review. PubMed PMID: 17558426.

Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat Immunol. 2002 Feb;3(2):196-200. Epub 2002 Jan 22. PubMed PMID: 11812998.

Henriksen-Lacey M, Bramwell VW, Christensen D, Agger EM, Andersen P, Perrie Y. Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. J Control Release. 2010 Mar 3;142(2):180-6. doi: 10.1016/j.jconrel.2009.10.022. Epub 2009 Oct 26. PubMed PMID: 19874860.

Huleatt JW, Jacobs AR, Tang J, Desai P, Kopp EB, Huang Y, Song L, Nakaar V, Powell TJ. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. Vaccine. 2007 Jan 8;25(4):763-75. Epub 2006 Aug 22. PubMed PMID: 16968658.

Jennings GT, Bachmann MF. Immunodrugs: therapeutic VLP-based vaccines for chronic diseases. Annu Rev Pharmacol Toxicol. 2009;49:303-26. doi: 10.1146/annurev-pharmtox-061008-103129. Review. PubMed PMID: 18851703.

Justin RA, Mallapragada SK. Enhancing the immune response through next generation polymeric vaccine adjuvants. Technology 02, 1. 2014.

Kensil CR, Wu JY, Soltysik S. Structural and immunological characterization of the vaccine adjuvant QS-21. Pharm Biotechnol. 1995;6:525-41. Review. PubMed PMID: 7551234.

Klinman DM, Klaschik S, Sato T, Tross D. CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases. Adv Drug Deliv Rev. 2009 Mar 28;61(3):248-55. doi: 10.1016/j.addr.2008.12.012. Epub 2009 Jan 14. Review. PubMed PMID: 19272313.

Lambrecht BN, Kool M, Willart MA, Hammad H. Mechanism of action of clinically approved adjuvants. Curr Opin Immunol. 2009 Feb;21(1):23-9. doi: 10.1016/j.coi.2009.01.004. Epub 2009 Feb 24. Review. PubMed PMID: 19246182.

Lee CC, Avalos AM, Ploegh HL. Accessory molecules for Toll-like receptors and their function. Nat Rev Immunol. 2012 Feb 3;12(3):168-79. doi: 10.1038/nri3151. Review. PubMed PMID: 22301850; PubMed Central PMCID: PMC3677579.

Levitz SM, Golenbock DT. Beyond empiricism: informing vaccine development through innate immunity research. Cell. 2012 Mar 16;148(6):1284-92. doi: 10.1016/j.cell.2012.02.012. Review. PubMed PMID: 22424235; PubMed Central PMCID: PMC3308125.

Lövgren Bengtsson K, Morein B, Osterhaus AD. ISCOM technology-based Matrix M™ adjuvant: success in future vaccines relies on formulation. Expert Rev Vaccines. 2011 Apr;10(4):401-3. doi: 10.1586/erv.11.25. Review. PubMed PMID: 21506635.

MacLennan IC, Toellner KM, Cunningham AF, Serre K, Sze DM, Zúñiga E, Cook MC, Vinuesa CG. Extrafollicular antibody responses. Immunol Rev. 2003 Aug;194:8-18. Review. PubMed PMID: 12846803.

Mannhalter JW, Neychev HO, Zlabinger GJ, Ahmad R, Eibl MM. Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake and antigen presentation. Clin Exp Immunol. 1985 Jul;61(1):143-51. PubMed PMID: 3876178; PubMed Central PMCID: PMC1577243.

Manolova V, Flace A, Bauer M, Schwarz K, Saudan P, Bachmann MF. Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol. 2008 May;38(5):1404-13. doi: 10.1002/eji.200737984. PubMed PMID: 18389478.

Marrack P, McKee AS, Munks MW. Towards an understanding of the adjuvant action of aluminium. Nat Rev Immunol. 2009 Apr;9(4):287-93. doi: 10.1038/nri2510. Review. PubMed PMID: 19247370; PubMed Central PMCID: PMC3147301.

Mbow ML, De Gregorio E, Valiante NM, Rappuoli R. New adjuvants for human vaccines. Curr Opin Immunol. 2010 Jun;22(3):411-6. doi: 10.1016/j.coi.2010.04.004. Epub 2010 May 11. Review. PubMed PMID: 20466528.

McHeyzer-Williams M, Okitsu S, Wang N, McHeyzer-Williams L. Molecular programming of B cell memory. Nat Rev Immunol. 2011 Dec 9;12(1):24-34. doi: 10.1038/nri3128. Review. PubMed PMID: 22158414; PubMed Central PMCID: PMC3947622.

Means TK, Hayashi F, Smith KD, Aderem A, Luster AD. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. J Immunol. 2003 May 15;170(10):5165-75. PubMed PMID: 12734364.

Miao EA, Warren SE. Innate immune detection of bacterial virulence factors via the NLRC4 inflammasome. J Clin Immunol. 2010 Jul;30(4):502-6. doi: 10.1007/s10875-010-9386-5. Epub 2010 Mar 27. PubMed PMID: 20349122; PubMed Central PMCID: PMC2993241.

Miyaji EN, Carvalho E, Oliveira ML, Raw I, Ho PL. Trends in adjuvant development for vaccines: DAMPs and PAMPs as potential new adjuvants. Braz J Med Biol Res. 2011 Jun;44(6):500-13. Epub 2011 May 13. Review. PubMed PMID: 21584443.

Morefield GL, Sokolovska A, Jiang D, HogenEsch H, Robinson JP, Hem SL. Role of aluminum-containing adjuvants in antigen internalization by dendritic cells in vitro. Vaccine. 2005 Feb 18;23(13):1588-95. PubMed PMID: 15694511.

Morel S, Didierlaurent A, Bourguignon P, Delhaye S, Baras B, Jacob V, Planty C, Elouahabi A, Harvengt P, Carlsen H, Kielland A, Chomez P, Garçon N, Van Mechelen M. Adjuvant System AS03 containing α-tocopherol modulates innate immune response and leads to improved adaptive immunity. Vaccine. 2011 Mar 16;29(13):2461-73. doi: 10.1016/j.vaccine.2011.01.011. Epub 2011 Jan 20. PubMed PMID: 21256188.

Neumann S, Burkert K, Kemp R, Rades T, Rod Dunbar P, Hook S. Activation of the NLRP3 inflammasome is not a feature of all particulate vaccine adjuvants. Immunol Cell Biol. 2014 Jul;92(6):535-42. doi: 10.1038/icb.2014.21. Epub 2014 Apr 1. PubMed PMID: 24687021.

O'Hagan DT, Singh M. Microparticles as vaccine adjuvants and delivery systems. Expert Rev Vaccines. 2003 Apr;2(2):269-83. Review. PubMed PMID: 12899577.Singh and O'Hagan, 2003

Opie EL, Freund J. AN EXPERIMENTAL STUDY OF PROTECTIVE INOCULATION WITH HEAT KILLED TUBERCLE BACILLI. J Exp Med. 1937 Nov 30;66(6):761-88. PubMed PMID: 19870697; PubMed Central PMCID: PMC2133531.

Pardoll DM. Spinning molecular immunology into successful immunotherapy. Nat Rev Immunol. 2002 Apr;2(4):227-38. Review. PubMed PMID: 12001994.

Pashine A, Valiante NM, Ulmer JB. Targeting the innate immune response with improved vaccine adjuvants. Nat Med. 2005 Apr;11(4 Suppl):S63-8. Review. PubMed PMID: 15812492.

Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunol Cell Biol. 2004 Oct;82(5):488-96. Review. PubMed PMID: 15479434.

Plotkin S., Orenstein W., Offit P. (2012) "Vaccines: expert consult" 6th edition, ElsevierPubMed PMID: 22566931; PubMed Central PMCID: PMC3342073.

Pulendran B. Modulating vaccine responses with dendritic cells and Toll-like receptors. Immunol Rev. 2004 Jun;199:227-50. Review. PubMed PMID: 15233738.

Pulendran B., Ahmed R. (2006) "Translating innate immunity into immunological memory: implications for vaccine development." Cell 124(4):849-63

Ramon G. (1925) “Sur l’augmentation anormale de l’antitoxine chez les chevaux producteurs de sérum antidiphtérique” Bull Soc Centr Med Vet 101:227-234

Rappuoli R, Black S, Lambert PH. Vaccine discovery and translation of new vaccine technology. Lancet. 2011 Jul 23;378(9788):360-8. doi: 10.1016/S0140-6736(11)60440-6. Epub 2011 Jun 12. PubMed PMID: 21664687.

Rappuoli R, Mandl CW, Black S, De Gregorio E. Vaccines for the twenty-first century society. Nat Rev Immunol. 2011 Nov 4;11(12):865-72. doi: 10.1038/nri3085. Review. Erratum in: Nat Rev Immunol. 2012 Mar;12(3):225. PubMed PMID: 22051890.

Reed SG, Bertholet S, Coler RN, Friede M. New horizons in adjuvants for vaccine development. Trends Immunol. 2009 Jan;30(1):23-32. doi: 10.1016/j.it.2008.09.006. Epub 2008 Dec 6. Review. PubMed PMID: 19059004.

Reed SG, Orr MT, Fox CB. Key roles of adjuvants in modern vaccines. Nat Med. 2013 Dec;19(12):1597-608. doi: 10.1038/nm.3409. Epub 2013 Dec 5. Review. PubMed PMID: 24309663.

Scheiermann J, Klinman DM. Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. Vaccine. 2014;32(48):6377-6389. doi:10.1016/j.vaccine.2014.06.065.

Schijns VE, Lavelle EC. Trends in vaccine adjuvants. Expert Rev Vaccines. 2011 Apr;10(4):539-50. doi: 10.1586/erv.11.21. Review. PubMed PMID: 21506650.

Schroder K, Tschopp J. The inflammasomes. Cell. 2010 Mar 19;140(6):821-32. doi: 10.1016/j.cell.2010.01.040. Review. PubMed PMID: 20303873.

Schwendener RA. Liposomes as vaccine delivery systems: a review of the recent advances. Therapeutic Advances in Vaccines. 2014;2(6):159-182. doi:10.1177/2051013614541440.

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. Erratum in: J Immunol. 2009 Jan 1;182(1):726. PubMed PMID: 18390722.

Shakya AK, Nandakumar KS. Applications of polymeric adjuvants in studying autoimmune responses and vaccination against infectious diseases. J R Soc Interface. 2013 Feb;10(79):20120536. doi: 10.1098/rsif.2012.0536. Review. PubMed PMID: 23173193; PubMed Central PMCID: PMC3565688.

Sjölander A, Cox JC, Barr IG. ISCOMs: an adjuvant with multiple functions. J Leukoc Biol. 1998 Dec;64(6):713-23. Review. PubMed PMID: 9850152.

Sjölander S, Drane D, Davis R, Beezum L, Pearse M, Cox J. Intranasal immunisation with influenza-ISCOM induces strong mucosal as well as systemic antibody and cytotoxic T-lymphocyte responses. Vaccine. 2001 Jul 16;19(28-29):4072-80. PubMed PMID: 11427284.

Sun HX, Xie Y, Ye YP. Advances in saponin-based adjuvants. Vaccine. 2009 Mar 13;27(12):1787-96. doi: 10.1016/j.vaccine.2009.01.091. Epub 2009 Feb 7. Review. PubMed PMID: 19208455.

Tacken PJ, de Vries IJ, Torensma R, Figdor CG. Dendritic-cell immunotherapy: from ex vivo loading to in vivo targeting. Nat Rev Immunol. 2007 Oct;7(10):790-802. Review. PubMed PMID: 17853902.

Tandrup Schmidt S, Foged C, Korsholm KS, Rades T, Christensen D. Liposome-Based Adjuvants for Subunit Vaccines: Formulation Strategies for Subunit Antigens and Immunostimulators. Pharmaceutics. 2016 Mar 10;8(1). pii: E7. doi: 10.3390/pharmaceutics8010007. Review. PubMed PMID: 26978390; PubMed Central PMCID: PMC4810083.

Tritto E, Mosca F, De Gregorio E. Mechanism of action of licensed vaccine adjuvants. Vaccine. 2009 May 26;27(25-26):3331-4. doi: 10.1016/j.vaccine.2009.01.084. Epub 2009 Feb 5. PubMed PMID: 19200813.

Tross D, Klinman DM. Effect of CpG oligonucleotides on vaccine-induced B cell memory. J Immunol. 2008 Oct 15;181(8):5785-90. PubMed PMID: 18832738; PubMed Central PMCID: PMC2562272.

Wagner TL. et al., 1999. Modulation of TH1 and TH2 cytokine production with the immune response modifiers, R-848 and imiquimod. Cell. Immunol. 191, 10, 1999.

Wen Y, Shi Y. Alum: an old dog with new tricks. Emerg Microbes Infect. 2016 Mar 23;5:e25. doi: 10.1038/emi.2016.40. Review. PubMed PMID: 27004761; PubMed Central PMCID: PMC4820675.

Wen ZS, Xu YL, Zou XT, Xu ZR. Chitosan nanoparticles act as an adjuvant to promote both Th1 and Th2 immune responses induced by ovalbumin in mice. Mar Drugs. 2011;9(6):1038-55. doi: 10.3390/md9061038. Epub 2011 Jun 14. PubMed PMID: 21747747; PubMed Central PMCID: PMC3131560.

Wilson-Welder JH, Torres MP, Kipper MJ, Mallapragada SK, Wannemuehler MJ, Narasimhan B. Vaccine adjuvants: current challenges and future approaches. J Pharm Sci. 2009 Apr;98(4):1278-316. doi: 10.1002/jps.21523. Review. PubMed PMID: 18704954.

Wilson-Welder JH, Torres MP, Kipper MJ, Mallapragada SK, Wannemuehler MJ, Narasimhan B. Vaccine adjuvants: current challenges and future approaches. J Pharm Sci. 2009 Apr;98(4):1278-316. doi: 10.1002/jps.21523. Review. PubMed PMID: 18704954.

World Health Organization. Global Vaccine Action Plan 2011-2020. Geneva: WHO; 2013