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Plant Genes and Plant Proteins as Adjuvants in Cancer Vaccination

Silvia Massa • Rosella Franconi*

ENEA, Italian National Agency for New Technologies, Energy and Sustainable Economic Development, C.R. Casaccia, UT BIORAD-FARM; Rome, Italy

Corresponding author: * **rosella.franconi@enea.it**

ABSTRACT

The use of plant products as anti-microbial and immunostimulants has a traditional history and has contributed to the development of important therapeutic drugs used in modern medicine. The anti-cancer properties demonstrated so far by some plant-derived immunostimulants rely on the modulation of non-specific immune responses and do not directly affect immune memory cells. However, it should not be unexpected to find some plant-derived components being able to stimulate antigen-specific cell-mediated immunity in the context of vaccines. The identification of novel adjuvants would give further potential to the development of improved therapeutic anti-cancer vaccines, able to promote better presentation or immunogenicity of tumour antigens and to improve trafficking of effector T-cell populations to non-inflamed tumour sites. Among plant products with effects on microbes and immunity, beside many small secondary metabolites, there are also proteins and peptides and most of them are involved in the plant defence against pathogens and invading organisms. We will present data from our work on the use of plant compounds (as extracts, proteins or DNA sequences) as sources of innovative immunostimulants in the context of therapeutic vaccination of Human Papilloma Virus (HPV)-associated tumours. Further studies are required to determine the mechanism of action by which plant extracts and their active compounds (including secondary metabolites, proteins and peptides) exert their anti-cancer and adjuvant activity for their exploitation in therapeutic vaccines. Moreover, new *in vivo* and *in vitro* approaches to isolate novel adjuvant activities are needed.

Keywords: immune-stimulation, immune-therapy, ribosome inactivating proteins, therapeutic vaccine, tumour immunity
Abbreviations: **APC**, antigen presenting cell; **CTL**, cytotoxic T lymphocyte; **DC**, dendritic cell; **DTH**, delayed-type hypersensitivity; **HPV**, Human Papillomavirus; **HSP**, heat-shock protein; **IgG**, immunoglobulin; **KLH**, Keyhole Limpet Hemocyanin; **NK**, natural killer cell; **NLR**, NOD-like receptor; **PAMP**, pathogen-associated molecular pattern; **PRR**, pathogen-recognition receptor; **RIP**, ribosome inactivating protein; **RLR**, retinoic acid inducible gene 1 like receptor; **TA**, tumour antigen; **Th1/Th2**, helper T (Th) cells immunity type 1 or 2; **TLR**, Toll-like receptor; **Treg**, regulatory T cell

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INTRODUCTION

The world has more than 300,000 species of higher plants and, according one recent estimate, more than 85% of them have not been evaluated systematically for their chemical composition and medical application (Pieters and Vlietinck 2005). Indeed, the use of plant products for therapeutic purposes has an ancient history, having been extensively utilised by ancient civilisations of Mesopotamia, Egypt, Greece, India and China (Newman *et al*. 2000; Wiseman 2002; Petrovska 2012). During the 20th century the modern medicine with vaccination, antibiotics and improvements in medical technology, contributed to a decline in botanical medicinal use. Nevertheless, a high percentage (> 60%) of the world's population uses botanic drugs to combat health problems (Farnsworth 1990). In Western countries, botanical medicinal consumption is on the increase for several reasons such as inability of modern medicine to effectively treat certain diseases (e.g. AIDS, malaria, cancer), need to contrast the spreading of antibiotic resistances among bacteria, preference towards natural alternatives (perceived as safe and effective) and willingness to self-medicate (Ernst and Pittler 2002; Schneiderman *et al*. 2003).

Many important therapeutic drugs used in modern medicine derive from plants: almost 50% of the synthetic medicines derive from phytochemicals and almost 30% of all pharmaceuticals approved by the US Food and Drug Administration (FDA) have a botanical origin, with digoxin,

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morphine, salbutamol and aspirin as successful examples (De Smet 1997). Among these, aspirin (derived from the active extract of the bark of the willow tree, called salicin), initially appreciated for its pain-relieving properties, recently has found an even more successful application, being repurposed for its cardiovascular protection activity. Repositioning might be applied to other plant-derived bioactive compounds, opening the field to new applications, such as cancer treatment.

Indeed, many of the compounds we use today for cancer treatment were initially discovered in plants. The widely-used drugs vinblastine and vincristine (anti-mitotic compounds used to treat leukemia and other cancers) were isolated from the Madagascar periwinkle (*Catharanthus roseus*) in the 1950s. The breast, ovarian, and lung cancer treatment paclitaxel (another mitosis inhibitor) was found in the bark of the Pacific yew tree (*Taxus brevifolia*) in 1967. Etoposide phosphate (an anti-tumour agent that disrupts proper DNA unwinding) was derived from podophyllotoxin, produced by the mayapple (*Podophyllum peltatum*) (Cragg and Newman 2005).

An increasing research interest for plant-derived compounds with immunomodulatory effects has emerged recently and has led to the study of the activity of plant compounds, particularly in the search for new-generation vaccine adjuvants (Kumar *et al.* 2011; Licciardi and Underwood 2011). Although, in fact, subunit vaccines offer great perspectives, their development is impaired by poor immunogenicity and/or inability to be properly presented to the immune system. For these reasons, besides the identification of the essential target, many efforts have to be spent for the improvement of treatment efficacy. Therefore, substances able to improve the immune response against the antigen (adjuvants) are fundamental to strengthen the efficacy of the active principle (antigen) in the vaccine. Despite current research efforts, aluminum salts (the first adjuvant to be described) still remain the standard for human use since only few others have been shown to be effective and safe. Consequently, there is an increasing need to develop novel vaccine adjuvants able to augment antigen-specific immunity.

Bioactive plant compounds could provide novel adjuvants that could give a major contribution to the vaccine industry for the development of new formulations for protective and therapeutic vaccines or immunotherapies (i.e. against cancer as well as chronic infectious, inflammatory, autoimmune, degenerative and metabolic diseases) and may offer advantages over current practices (Licciardi and Underwood 2011). Among plant compounds, beside small organic molecules with immunostimulant properties, also bioactive proteins and peptides are of interest: these could be genetically engineered and produced as recombinant molecules or fused to tumour antigens (TAs) to obtain improved genetic or protein therapeutic vaccines.

In addition to the identification of immune response potentiators, novel platforms for the development and production of cheaper, safer and more potent biopharmaceuticals are important. Over the last few years, plants have shown more than just 'potential' for the production of recombinant vaccines (Rybicki 2009) and the first therapeutics, including cancer therapeutics (Pujol *et al.* 2007; Kaiser 2008) are approaching approval (Horn 2012). In addition, plant-derived and 'green' contained systems represent important biotechnological approaches for the improved and sustainable production of valuable therapeutic secondary metabolites, as well (Franconi *et al.* 2010a).

The use of plants both as a source of immunostimulating genes/proteins and as biofactories promises to turn traditional medicines into modern drugs through the identification of novel compounds, the elucidation of the mechanisms responsible for immune response modulation able to augment vaccination efficacy, and to the production of innovative and more targeted plant-based medicines for the treatment of human diseases.

This review, will give an overview on the topic of the available plant-derived bioactive molecules in the field of vaccine adjuvant development. Moreover, we will show applications of this approach to the immunotherapy of the Human Papilloma Virus (HPV)-associated tumours (Bergot *et al.* 2011). HPV is responsible for cervical cancer in women and globally of about 5% of all cancers for which neither specific pharmacological nor immunotherapy are available: plant-derived remedies might represent the next frontier and, hopefully, one possible solution.

IMMUNOMODULATION AND ADJUVANTS

Current options for vaccine adjuvants

Immunomodulation is the regulation of human immune system, essential for protecting against the damaging effects of pathogens and cancer by agents that activate or suppress its function. Activating the immune system for therapeutic benefits in cancer has long been a goal in immunology and oncology. The system comprises innate (non-specific) and acquired (specific) immunity. Natural killer (NK) cells, complement system, macrophages, antigen presenting cells (APCs), with dendritic cells (DCs) representing specialised 'professional' antigen-presenting cells, and neutrophils make up the innate immune system, and mounts an immediate non-specific response to foreign microbial agents but also against cancer cells.

If invaders or exogenous antigens by-pass this primary defence, the acquired immune response, comprising humoral and cell-mediated components, will act with specific cytokines secretion which determine the differentiation of helper T (Th) cells into Th1 (cell-mediated immunity) or Th2 cells, and B-cells to give immunoglobulin subtypes (humoral immunity).

Vaccines or any antigens are defined as specific immunostimulators, while non-specific immunostimulators are those that stimulate components of the immune system irrespective of antigenic specificity, such as adjuvants. Therefore, broadly defined, adjuvants are compounds that activate innate immunity and enhance the potency of the antigen-specific immune response, representing essential components of an efficacious vaccine.

Increased understanding of immunology and particularly of the innate immune system is informing vaccine adjuvant research and consequently driving the development of novel and specifically directed vaccine adjuvant strategies. The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) by means of pathogen-recognition receptors (PRRs), which include the Toll-like receptors (TLRs), expressed on the cell surface and endosomal compartments, as well as cytoplasmic associated receptors such as NOD-like receptors (NLRs) and retinoic acid inducible gene 1 like receptors (RLRs) (Kumar *et al.* 2009).

Adjuvants commonly found in modern vaccines are generally classified in two major classes:

• *Vehicles*: defined as components that present vaccine antigens to the immune system in an optimal manner, including controlled release and depot delivery systems to increase the specific immune response to the antigen. Examples include: mineral salts, emulsions, liposomes, virosomes, biodegradable polymer microspheres, Quil-A, QS-21, and immune stimulating complexes like ISCOM and ISCOMATRIX™.

• *Immunostimulants:* defined as components that directly engage the immune system to increase responses to antigens (e.g. TLR/NLR/RLR ligands, cytokines, saponins, and bacterial exotoxins).

Most adjuvants present in approved human vaccines against infectious diseases such as Aluminum salts, MF59 (Novartis) and AS03 (GlaxoSmithKline), belong to the first category. Aluminum salts have been used as an adjuvant

with great success for almost a century and have been particularly effective at promoting protective humoral immunity. However, alum is not optimally effective for diseases where cell-mediated immunity is required (i.e. against cancer). On the other hand, MPL®, that belongs to the second class of adjuvants, is the first and only TLR ligand in licensed human vaccines (Fendrix® for HBV and Cervarix® for HPV contain AS04, a combination of alum and MPL®). It is a potent stimulator of T cell and antibody responses being a derivative of the lipopolysaccharide (LPS) from *Salmonella minnesota* (Didierlaurent *et al.* 2009).

The development of new adjuvants has been quite slow essentially because adjuvants do not receive FDA approval as stand-alone products, but as part of a registered vaccine adjuvant–antigen combination, and because of the high cost of developing novel adjuvant formulations, together with uncertainties regarding regulatory approval.

Current options for vaccine adjuvants in the case of cancer immunotherapy

In the case of cancer and chronic infectious diseases, it may be difficult to develop effective immune-therapy without blocking immune regulatory pathways, which impede cell-mediated responses. In fact, in this regard, adjuvants are necessary to boost the desired immune response to tumour antigens (TAs) that are poorly immunogenic and that, when administered alone without appropriate immunostimulatory signals, elicit anergic or regulatory T cells (Treg). A necessary step to overcome the immune-suppressing barriers of the tumour milieu is the potent triggering of innate immunity, leading to the recruitment, activation, and maturation of antigen presenting cells such as dendritic cells (DCs), in turn activating pre-existing or priming a vigorous TA-specific adaptive cellular immune response, facilitating the induction of cytotoxic T lymphocytes (CTLs) (CD8+ T cell) that can traffic to and lyse malignant cells (Palena and Schlom 2010).

In 2011, the FDA approved some pioneering cancer vaccines: *Provenge*® (Dendreon Corporation), a patient-specific cellular immunotherapy for prostate cancer, and *Yervoy-Ipilimumab* (Bristol-Meyes Squibb), a human monoclonal antibody against CTLA-4 for melanoma. In September of the same year, the Cuban medical authorities launched the sales of the world's first therapeutic vaccine against lung cancer, *CimaVax* (developed by the Centre of Molecular Immunology, CIM), based on the epidermal growth factor (EGF) formulated with ISA51V (Montanide).

These data demonstrate the renewed interest and investment in the development of new therapeutic cancer vaccine candidates, notwithstanding a two-decade track record of failure, the reason for which are mainly: ineffective priming of tumour-specific T-cells by available formulations (vaccine design mimic infectious antigen-based formulations); effects of tumour microenvironment (i.e. Treg, cytokines IL-10 and TGF-b) that not only supports tumour growth and metastasis but also reduces potential adaptive immunity to tumour antigens, lack of suitable animal models, single modality of therapy. Advances in understanding how tolerance, immunity and immunosuppression regulate anti tumour immune responses, together with the advent of new targeted compounds and immunotherapeutics is leading to a strategic use of vaccine technology in order to get a proper antigen 'education', inhibition of immune inhibitors and enhancement of immune functions to obtain a durable and long-lasting response in cancer patients (Mellman *et al*. 2011). Currently, there are several ongoing clinical studies that utilize defined molecular adjuvants that show great promise in therapeutic vaccines against several cancers (Dubensky and Reed 2010).

Among these, the live-attenuated tuberculosis vaccine Bacille Calmette-Guérin (BCG), derived from *Mycobacterium bovis* interacts with TLR-2, TLR-4 as well as with NLR-2. With its FDA approval in 1990 for the treatment of bladder cancer, BCG was the only therapeutic cancer adjuvant until the recent approval of Provenge. Through cross-presentation mechanisms resulting from the induction of innate immunity and activation of NK cells, BCG and agonists like the TLR-9 agonist CpG can elicit TA-specific adaptive immunity. BCG has also been used to adjuvant cell based vaccines, and is a component of Melacine, approved in Canada for treatment of melanoma.

Heat-shock proteins (HSPs) and Keyhole Limpet Hemocyanin (KLH), have been used as non-specific adjuvant conjugated with recombinant protein or peptide-based vaccines. HSPs (i.e. HSP70 and Gp96) enhance vaccine potency by chaperoning antigenic peptides to MHC class I molecules at the cell surface for presentation to lymphocytes (Tsan and Gao 2009). KLH is a strongly immunogenic high molecular weight metalloprotein that elicits a mitogen-activated protein kinase (MAPK)-dependent delayed type hypersensitivity (DTH) response (Engstrom *et al.* 2009) used for non-Hodgkins lymphoma and glioblastoma (Dubensky and Reed 2010).

Many cytokines that are produced in response to activation of innate immunity are also used as recombinant proteins, fusion partners with selected TAs and co-expressed with TAs in gene-based cancer vaccines. For example, interferon-alpha2b provide benefit as an adjuvant therapy for high-risk melanoma (Tarhini and Kirkwood 2009).

Various TLR agonists alone or in combination, have shown to significantly enhance vaccine potency, when added as microparticles/nanoparticles or liposomes to vaccine formulations. These include TLR-3 (poly I:C), TLR-4 (monophosphoryl lipid A; MPL), TLR-5 (flagellin), TLR-7 (Aldara, Imiquimod, approved for treatment of actinic keratosis, basal cell carcinoma and genital warts), TLR-7/8 (Resiquimod), and TLR-9 (CpG) (Zhu *et al.* 2008; Warshakoon *et al*. 2009). A synthetic TLR-4 agonist being developed with adjuvant properties equal or superior to MPL® (already discussed) is RC-529 (GSK, DynaVax), which is licensed for a HBV vaccine in Europe. These are only some examples of TLR agonists.

Based on the success of these and other adjuvants that activate innate immunity, the development and clinical evaluation of novel small molecule adjuvants specific for designated TLRs and NLRs is a highly active and competitive area of the pharmaceutical industry.

Immunomodulation and adjuvant activity by plant-derived compounds

The use of plant-derived products as candidate vaccine adjuvants is one way to identify important immunologically-active compounds. This has been the focus of studies in our laboratory and elsewhere (Underwood *et al.* 2009; Licciardi and Underwood 2010).

1. Whole plant extracts and fractions

The use of plant extracts for their immunostimulatory properties has a traditional history (Kumar *et al.* 2011), although the isolation of the active principles involved did not gain momentum until the 19th century. Several studies about Traditional Chinese Medicine (TCM) herbs have been performed to elucidate, in some detail, the immunomodulatory and antimicrobial effects. For example, Tan and Vanitha (2004) reviewed the chemicals present in seven TCM plants, their respective biological activities and their effects on some biochemical parameters of the immune system. A broad range of different plant-derived extracts, like the Neem tree leaf preparation, a compound of Chinese herbal medicinal ingredients (Wang *et al*. 2005) and the Rb1 fraction of ginseng (Rivera *et al*. 2005) has been evaluated for adjuvanticity. Some of these extracts together with molecules like triterpenoids are as effective as Freund's complete or incomplete adjuvant, and able to induce balanced Th1 and Th2 responses even towards TAs (Baral *et al*. 2005; Kumar *et al.* 2012).

2. Plant saponins

Most known plant-derived adjuvants are saponins, secondary metabolites characterized by their strong foam-forming properties in aqueous solution and comprising a lipophylic part (sapogenin). They are formed by a steroid or other triterpene aglycon decorated with one or more hydrophilic glycoside moieties. Plants produce a wide variety of saponins that exhibit many different biological activities, from antimicrobial to cytotoxic and antitumoral, which are on the basis of many uses of plants in traditional medicine (Sparg *et al.* 2004). On the basis of their common use within the food and beverage industries, with no documented toxicity in humans at the present levels of consumption, food-grade saponins have also been proposed as adjuvants for use with plant-made vaccines (Kirk *et al*. 2004), like the QS-21 acylated 3,28-o-bisdesmonic triterpene saponin extracted from the bark of the tree *Quillaja saponaria* Molina of South America. Basically, the interest to this natural surfactant derives from its high water solubility and stability (days) in combination with the antigen. Both in animal models and in human trials, QS-21 has been shown to enhance both Th1 and Th2 responses (Moore *et al.* 1999). However, serious drawbacks have limited its use as an adjuvant in human vaccination, such as high toxicity, haemolytic effect and instability (Waite *et al*. 2001) although this has not precluded its use in experimental and commercial veterinary vaccines such as foot-and-mouth disease (Cox *et al.* 1999; Borja-Cabrera *et al.* 2004). Also variants of natural and semi-synthetic saponins are used: immune stimulating complexes like GPI-0100, ISCOM and ISCOMATRIX™ (Marciani *et al*. 2003; da Silva *et al.* 2005). The ISCOM adjuvant formulations provide evidence for the use of plant-derived materials in the stimulation of effective immune responses.

Of relevance for the development of plant mucosal vaccines is the presence of possible saponin-based immunomodulators in edible crops, which could eventually act as endogenous adjuvants of plant-based vaccines. Potato contains mainly α-solanine and α-chaconine, whereas green tomatoes are rich in α-tomatine. Tomatine adjuvant is a highly effective immunostimulator, being capable of generating anti-ovalbumin humoral responses in mice after a single immunization. Interestingly, these responses were achieved in the absence of toxicity (i.e., inflammation) (Rajananthanan *et al*. 1999). Subsequently, tomatine adjuvant was shown to enhance a cellular immune response (Heal *et al*. 2001; Morrow *et al*. 2004).

Plant polysaccharides as low-toxicity adjuvants: Natural adjuvants that combine potent immuno-stimulatory activity with low toxicity are currently undergoing clinical trials. As an example, Canberra's Vaxine is trying a potent, plant-derived adjuvant, Advax, a modified version of a compound called inulin, extracted from dahlia tubers (Petrovsky 2008). Inulin is a fructan that represents a reserve carbohydrate in some flowering plants also involved in adaptation to drought and cold stress (Hendry *et al.* 1993). Inulin is widely used in human nutrition and it is generally regarded as safe (GRAS) by the FDA and it is heat stable with a long shelf-life. Very good at stimulating both antibody and T-cell immunity (Petrovsky 2008), it does this by stimulating the immune system through activating the complement pathway with low toxicity (a paramount in an adjuvant since most adjuvants that elicit good T-cell immunity in mice are too toxic to be used in humans). Inulin adjuvant has been shown to improve vaccine responses in animal tests against hepatitis B (Cooper *et al*. 1991), malaria (Saul *et al.* 1992) and influenza in a spray freeze-dried preparation (Amorij *et al.* 2007). A Phase I trial of inulin as an adjuvant for an alternative HPV vaccine developed by Ian Frazer (University of Queensland, Australia) to protect against cervical cancer, confirmed it produced no adverse side-effects (Merck and Glaxo commercialise the HPV vaccine, using aluminum adjuvant and AS04, respectively).

A number of additional plant-derived polysaccharides have been assayed in recent years, such as *Lemna minor* apiogalacturonic peptin, *Actinidia eriantha* polysaccharide or, more recently, *Lycium barbarum* polysaccharide–protein complex. The latter complex has recently been shown to induce phenotypic and functional maturation of DCs with strong immunogenicity, which makes it a potent candidate adjuvant for the design of DC-based vaccines (Chen *et al.* 2009).

3. Plant proteins

It is interesting to observe how most of the plant molecules with proven immunomodulatory activity, from saponins to lectins, have endogenous defensive roles, particularly against herbivores. Noteworthy, among plant products with effects on human pathogens, immunity and cancer there are also plant peptides and proteins involved in the defence of plants against pathogens and invaders. Regardless of the existence of a biological rationale underlying the responsiveness of the human immune system to the defensive molecules produced by plants, this fact can be biotechnologically exploited in the context of vaccine adjuvants.

In particular, different plant and mushroom proteins are defence proteins or antipathogenic proteins in the sense that they protect plants and mushrooms from diseases caused by intruding pathogens and have shown *in vitro* or *in vivo* activity against pathogens that attack humans and against human diseases. As an example, some plant and mushroom antifungal proteins also exhibit modulation of non specific (innate) immunity patterns: mitogenic activity towards spleen cells, nitric oxide inducing activity toward macrophages, antiproliferative activity toward tumour cells, antibacterial activity, and inhibitory activity toward HIV-1 reverse transcriptase (Tan and Vanitha 2004). Antifungal proteins isolated from wild mushrooms or by the medicinal mushroom *Ganoderma lucidum* can inhibit human bacterial pathogen growth, HIV-1 reverse transcriptase, proliferation of HepG2 hepatoma cells and L1210 leukemia cells. Agocybin exhibits mitogenic activity toward mouse splenocytes. Some glycoproteins from the Neem tree leaf extracts were seen to restrict *in vivo* the growth of mice tumours by increased interferon-gamma secretion (Wang *et al.* 2005).

Plant lectins are a class of plant carbohydrate-binding proteins that are ubiquitously distributed in plants, animals and fungi. Plant lectins are being explored as potential antitumour drugs for their ability to induce programmed cell death in cancer cells (Fu *et al.* 2011). The interest of lectins in vaccine development comes from their unique ability to bind carbohydrate moieties of particularly M cells at the Peyer's patches of mucosal surfaces, whereby immune complexes and antigens in the gastrointestinal lumen are sampled and delivered to DCs that can migrate to the lymphoid tissue to interact with T and B cells. This property has led to postulate some plant lectins as possible antigen-delivery agents. The role of many lectins in plant defence is well established, acting as anti-nutrients and, therefore, protecting against herbivores through toxicity. Probably the best-studied lectin is ricin B. The lectin subunit binds to the cell surface and directs the entry of A subunit into the cell, the toxic A subunit inactivates ribosome activity blocking protein synthesis and causing cell death (Wool *et al.* 1992).

A possible use of plant lectins as mucosal adjuvants was seen and they were compared with Cholera Toxin (CT) as adjuvants for ovalbumin, a model antigen (Lavelle *et al.* 2001). Ricin B has been tested for delivery of antigens to the mucosal immune system. Fused to the green fluorescent protein (GFP), it was expressed in tobacco plants and hairy root cultures to test mucosal vaccine delivery/adjuvancy (Medina-Bolivar *et al.* 2003). Intranasal immunization of mice with purified ricin B–GFP triggered GFP-specific serum IgGs, comparable with that observed following GFP immunization with CT adjuvant. Also a ricin B N-terminus fusion with a peptide from simian rotavirus protein NSP490 stimulated a strong Th1 cell-mediated immune response

(Choi *et al*. 2006). More recently, ricin B–insulin fusions produced in edible plant tissues have also been reported as an adjuvant strategy for inducing oral tolerance against type 1 diabetes (Carter *et al.* 2010).

Plant lectins have also been used to target antigens to M cells of the Peyer's patches in microencapsulation strategies: lectinized nanoparticles could be a promising carrier–adjuvant for the targeted oral–mucosal immunization (Gupta *et al*. 2007). Recently, a new strategy for vaccination against *Helicobacter pylori* and *Campylobacter jejuni* was assayed involving the lectinization of whole-bacteria preparations (Chionh *et al*. 2009).

Although a physiological role of heat-shock proteins (HSP) in antigen presentation and immune response activation has not been directly demonstrated, their use as vaccine components is under clinical trial. The structure of plant-derived HSP70 (pHSP70), typical stress products, can be superimposed to the mammalian homologue and similarly to the mammalian counterpart, pHSP70-polypeptide complexes can activate the immune system. It has been shown that pHSP70 purified from plant tissues transiently expressing the influenza virus nucleoprotein are able to induce both the activation of major histocompatibility complex class I-restricted polyclonal T-cell responses and antibody production in mice of different haplotypes without the need of adjuvant co-delivery. These results indicate that pHSP70 derived from plants producing recombinant antigens may be used to formulate multiepitope vaccines (Buriani *et al.* 2012).

CASE STUDY: PLANT DERIVED ADJUVANTS FOR THERAPEUTIC VACCINATION OF HPV-ASSOCIATED CANCERS (EXTRACTS, PLANT GENE PRODUCTS)

HPV infection is becoming preventable through vaccination (Bryan 2007; Bergot *et al*. 2011). Nevertheless, immunetherapy is needed to exert immediate effects on lowering HPV-related infections and tumour incidence. Treatment of HPV-associated tumours and of cancer in general, should benefit from therapies that boost immune cell-mediated tumour defence mechanisms toward the relevant tumour antigens (for HPV, the early E6 and E7 proteins) (Pardoll 1998; Stevenson *et al.* 2004). Strategies to enhance therapeutic HPV vaccine efficacy have stimulated great interest in pre-clinical and clinical studies (Ma *et al*. 2010; Su *et al.* 2010). In particular, we focussed on plants as sources of innovative immune-stimulatory molecules to enhance HPV vaccination potency against the E7 antigen.

Plant-derived extracts as DCs modulating agents for cancer vaccines

In 2002, we described the ectopic expression of HPV-16 E7 protein in tobacco plants (*N. benthamiana*) using a viral vector based on *Potato Virus X* (PVX) (Franconi *et al.* 2002). We analysed the E7 expression also in the PVX hosts *N. rustica*, *N. tabacum*, *Chenopodium quinoa*, and the miniature *Lycopersicon esculentum* cultivar 'Micro-Tom', concluding that the E7 protein was expressed at highest levels in *N. benthamiana* (3-4 µg/g fresh leaves).

The foliar extract from *N. benthamiana* leaves containing the E7 protein was used to immunize mice with a vaccine dose containing 0.5 µg of plant-made recombinant E7. Control mice were immunized with 10 µg of *E. coli*-made recombinant His-E7 plus the saponin-derived adjuvant Quil-A. E7-specific humoral and cell-mediated immune responses were induced in the mice. The anti-tumour activity of the vaccine was evaluated by challenging vaccinated mice with C3 cells, an embryonic mouse cell line expressing the HPV-16 E7 oncoprotein. Both vaccine preparations inhibited the tumour growth in 40% of vaccinated mice, whereas the control mice and mice vaccinated with foliar extract from plants infected with wild type PVX were all tumour-affected 7 days after the challenge. The amount of E7 protein in the foliar extract was enhanced five-fold when the E7 gene was fused to a plant-derived signal sequence and, as a consequence, also the anti-tumour activity of this vaccine preparation was enhanced: 80% of mice immunized with the new extract (2.5 µg of E7 per dose) were tumour-free 50 days after challenge with tumour cells and the protection was associated with a strong Th1 cell response (Franconi *et al.* 2006). These results strongly suggested that an adjuvant-like activity was present in the foliar extract.

The adjuvant-like activity of the vaccine from *Nicotiana benthamiana* plant extract and the possible use plant extracts in the immunotherapy of HPV-related lesions were investigated using human DCs, antigen presenting cells which are major players in the induction of immune responses in the target tissues. DCs are critical regulators of immunity that operate at the interface between the innate and adaptive immune systems. DCs that reside in peripheral lymphoid tissues become activated following encounter and phagocytosis of antigen through engagement of cell-surface receptors (e.g. TLRs and CD14) with pathogenic molecules such as LPS (van Duin *et al*. 2006). Activated DCs interact with antigen-specific CD4+ T-lymphocytes and migrate to the infected site where the expression of costimulatory molecules on the cell surface is upregulated (e.g. CD80, CD40, CD86, and CTLA-4) to stimulate appropriate effector T-lymphocyte function (Banchereau *et al.* 2000). Given the integral role for DCs in the initiation of immune responses, experimental approaches based on the design of novel therapeutics that exploit DC function may provide effective alternatives to current immunotherapies.

The plant extract containing E7 was not toxic for the cells, did not influence E7 uptake into the cells, did not affect DC differentiation, but did induce DC maturation, as shown by the phenotypic expression of specific markers (Di Bonito *et al.* 2009). This effect was not caused by LPS but rather to the presence in the foliar extract of heat-resistant products mimicking the effect of LPS. Importantly, the E7-containing extract was able to prime naïve lymphocytes to produce an E7-specific T cell response. While this study gives some valuable insights about the immunomodulatory activity of the *N. benthamiana* plant extract in a preclinical model, it is fundamental to determine the chemical nature of the compounds responsible for the immunomodulatory properties of the *N. benthamiana* extracts (i.e., aggregates, viral RNA, protein post-translational modifications, lectins, HSPs, lipids) for a possible application in humans as well as to isolate the adjuvant compound(s) from *N. benthamiana*, that could thus be included in the list of immunostimulant plants.

Mutants of plant genes for developing immunostimulants for cancer vaccines

Plant production of purified candidate HPV therapeutic vaccines was also achieved by expressing a non-oncogenic E7 protein variant as a fusion with a protein of bacterial origin (0.5 mg/g fresh leaf tissue after purification) by using a 'second-generation viral vector' for transient plant expression (Massa *et al.* 2007). In studies carried out on large numbers of animals (50 per treatment), after two doses of purified plant-derived E7 tumour antigen (20 µg), very aggressive E7-expressing experimental tumours growth block was achieved by inducing a huge E7-specific T-cell response also in absence of any adjuvant (Venuti *et al.* 2009).

In a search of innovative immunostimulatory sequences with low clinical use constraints (i.e., possible auto-immune responses induced by proteins of human origin like Hsp70 and calreticulin for adverse auto-immunity, and tetanus toxoids for weakened response in tetanus-vaccinated individuals) able to enhance the potency of a genetic HPV vaccine, our first strategy was to fuse an attenuated HPV16 E7 (E7GGG) to the coat protein of PVX, a plant virus (Massa *et al.* 2008) a carrier that had been shown to increase CD4+ T cell immune response (by 'linked T cell help'), and to en-

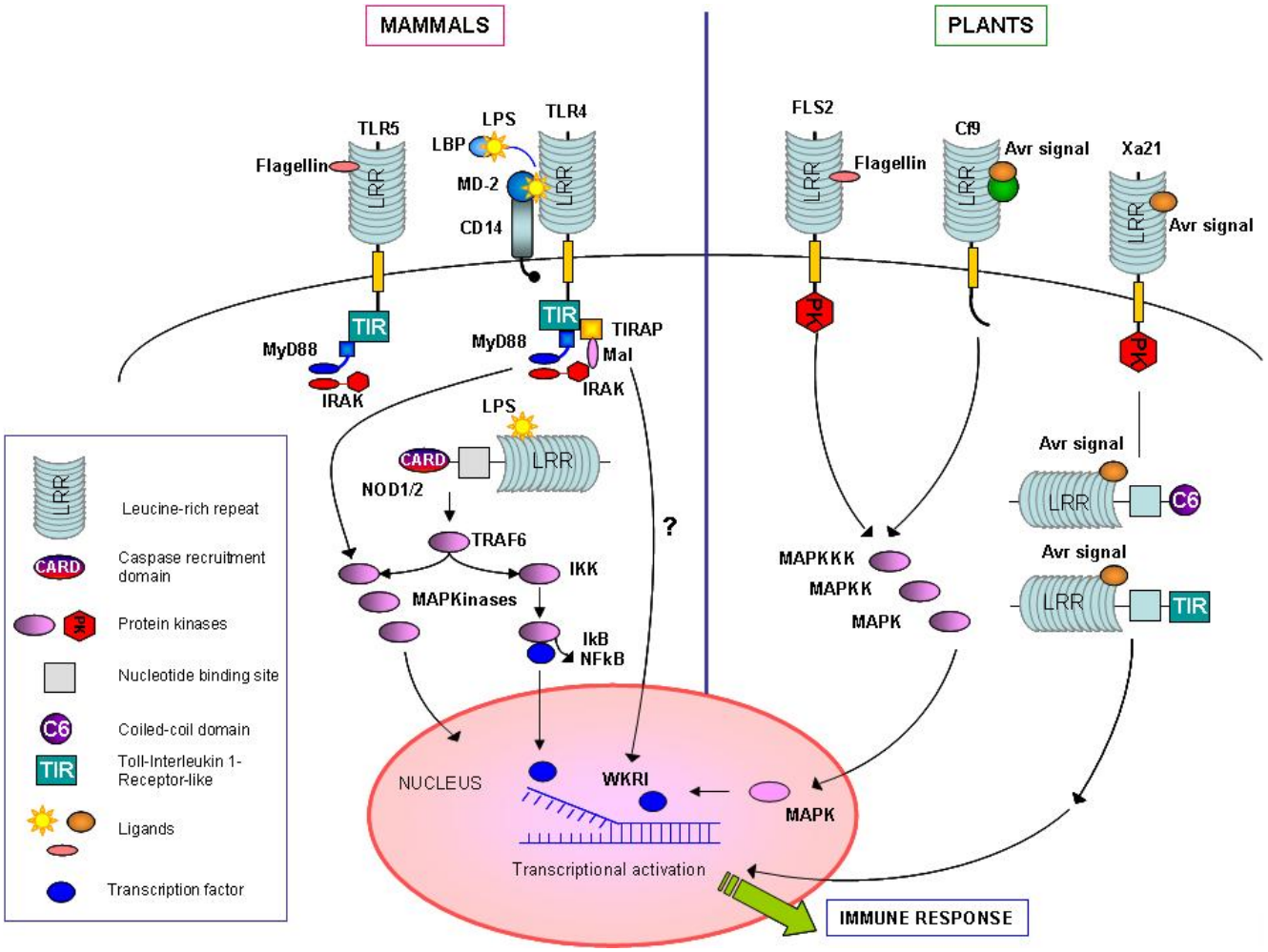

Fig. 1 Conservation of signalling pathways mediating the activation of innate immunity in mammals and plants. TLR4, TLR5, FLS2, and the plant resistance genes Cf9 and Xa21 exemplify transmembrane receptors for the recognition of pathogen-associated molecular patterns (PAMPs, like lipopoly-saccharide 'LPS' and flagellin) or avirulence signals (Avr). The LPS envelope of Gram-negative bacteria stimulates innate immunity in mammals. Upon recognition by LPS-binding protein (LBP), a complex with leucine-rich repeat (LRR) proteins CD14 and TLR4 (which contains a cytoplasmic TIR domain) is formed. Flagellin perception in mammals is mediated by TLR5. TLRs interact via adapter proteins like MyD88 (myeloid differentiation factor). Subsequently, a series of protein kinases from plants, including mitogen-activated protein kinases (MAPKs), mediate activation of transcription factors (nuclear factor-kB, NF-kB) through inactivation of the repressor protein inhibitors (IkB) and expression of immune response genes. In plants, various LRR-type proteins with similarity to CD14/TLR appear to be involved in pathogen defence activation. Avr9 is recognised by a high-affinity binding site. This complex interacts directly or indirectly with Cf9 and activate MAPKs. FLS2 and Xa21 are likely to transduce the pathogen signal through their cytoplasmatic protein kinase domain. Translocation of PAMP-activated plant MAPK into the nucleus has been demonstrated, where these enzymes are likely to contribute to the activation of the transcription factors of the WRKY type. Intracellular recognition of pathogen-derived molecules takes place in plants as well as in mammals. Intracellular recognition of LPS in mammals is mediated by the NBS (nucleotide binding site)-LRR receptors NOD1/2. In plants, R proteins recognizing Avr signals confer pathogen-specific immunity and have NBS-LRR as well (adapted from Ronald and Beutler 2010).

hance immunogenicity of silent or poor determinants (Gerloni *et al.* 2002; Savelyeva *et al.* 2003).

In a following study, a new strategy was developed by using plant proteins, the 'Ribosome inactivating proteins' (RIPs). RIPs are potent inhibitors of protein synthesis (through catalytic N-glycosidase activity on rRNAs) that accumulate in different organs of many plant species where they have a regulative and a defence role (Hartley and Lord 2004). Indeed, RIPs, are involved in the plant defence against pathogens and invading organisms (Stirpe 2004; Tan and Vanitha 2004; Fang *et al.* 2011).

Although cytotoxicity due to protein synthesis arrest was the first feature to be characterized and clinically exploited to build selective cell-killing agents of tumour, immune or nerve cells (Cheung *et al.* 2004; Stirpe 2004; Zarovni *et al.* 2009; Ng *et al.* 2010). RIPs show other biological properties, independent of catalytic activity, that could re-purpose these proteins to give contribute to the design of therapeutic anti-tumour vaccines. These activities include the ability to modulate non specific and innate immune functions affecting NK (Hajto *et al.* 1998), CD4+ and CD8+ T cells (Bhutia *et al.* 2009), cytokine production (Hajto *et al.* 1990; Bhutia *et al.* 2009), inflammation (Zhao *et al.* 1999) and apoptosis through multiple pathways (Stirpe 2004; Sikriwal *et al.* 2008; Li *et al.* 2009) that have been shown to lead to anti-tumour properties *in vivo* (Bhutia *et al.* 2009).

Due to conservation of molecular organization mediating the activation of innate immunity in mammals and plants (Ronald and Beutler 2010) (**Fig. 1**) and due to the innate immunity initiator role of the adaptive response, it might be found that some of these plant proteins might stimulate also (tumour) antigens-specific cell-mediated immunity (crucial for cancer resolution). In this sense plant proteins would really behave like adjuvants (agents that potentiate and possibly target specific immune response to an antigen, according to FDA definition).

Data from our experiments support this hypothesis. For the first time we reported that the fusion of HPV16 E7GGG gene with a saporin (the main RIP produced by *Saponaria officinalis*) non catalytic mutant (SAP-KQ) can lead to improvement of antigen-specific DTH, humoral and cell-

mediated immune responses resulting in enhanced anti-tumour effects against E7-expressing tumours in the context of DNA-based vaccination with respect to the E7GGG gene alone (Franconi *et al*. 2010b; Massa *et al*. 2011).

Results obtained upon transfection of mammalian cells with SAP-KQ/E7GGG gene fusions, indicated that fusion proteins containing SAP-KQ undergo a rapid degradation through the ubiquitin-proteasome pathway. This result was already found for the PVX coat protein fused at the C-terminus of E7GGG (Massa *et al.* 2008) and it could be not surprising in the case of SAP-KQ fusions since it has been proven that the A-chain of type II RIPs administered to cells, once entered the cytosol, are subjected to efficient protein quality control: they are recognized as misfolded proteins probably as a consequence of an interaction with the lipid cell membranes and are often easily targeted by proteasomes and ubiquitylated to be eliminated. The finding is particularly true for high lysine content RIPs (Hartley and Lord 2004) like saporins (10% of the aminoacids in saporins are lysine residues). In the end, this could contribute to a better processing of the E7GGG antigen fused to the SAP-KQ that could have contributed to the improved activity of the fusion DNA vaccine with respect to the E7GGG alone.

Nevertheless, further studies are needed to explore new mutants (Bonini *et al*. 2006) and to elucidate the immunological feature that may be related to the induction of local inflammation at the injection site or recruitment of NK cells and/or macrophages with inflammatory phenotype.

Heterologous prime-boost regimens

While the potency of adjuvanted protein vaccines has increased with the development of agonists, regimens that utilize multiple vaccine modalities may ultimately prove to be the most effective therapeutic strategies. The combination of gene-based vector modalities with protein compositions may be the best approach to both prime new CD4+ and CD8+ tumour antigen-specific T cell populations, as well as to boost pre-existing tumour-specific memory T cell populations.

Our immunogens are being tested following these heterologous vaccination regimens (plasmid DNA/viral vector/plant-expressed protein) to determine the efficacy of different prime-boost protocols. Indeed, although protection/recovery from HPV-related tumours is mainly cell-mediated (provided by plasmid DNA), a humoral antigen-specific systemic and mucosal response is also very important to elicit a protective immunity and a long-lasting memory response (followed to viral vector-based recombinants and/or protein boosts). Therefore, different immunisation protocols will contribute to determine the immune correlates of protection in tumour-cured versus non-cured animals.

CONCLUSIONS AND PERSPECTIVES

The molecular diversity of the plant kingdom offers a vast source of compounds with immune-stimulant and adjuvant activity, of which only a minute fraction has been characterized and exploited (Kinghorn *et al.* 2011). Active compounds derived from some plants were also found with unique features as TLRs agonists and antagonists, bringing about new hopes and evidences for the application of these naturally existed TLRs modulators, encouraging further work on the characterization of these compounds as promising drug candidates in TLRs-based therapy in the future (Liu *et al.* 2011).

In addition, in spite of significant amount of knowledge has been gained during the last decades about the biosynthetic capacity of plants and about the pathways leading to the formation of plant natural products, there is still a significant number of high value products, included adjuvants, which are obtained from natural sources due to lack of alternative resources (e.g. no chemical synthesis feasible). As an example, of the overexploitation of the *Q. saponaria* bark has caused important ecological damage and a considerable shortage of the available supplies. Thus, development of candidate adjuvants should focus on moving away from current, natural product based methods to the production of sustainable adjuvant formulations.

Until now, chromatographic separation of plant extracts and high-throughput screening has constituted the main tool for plant adjuvant discovery (Koehn 2008). However, plant metabolomics and transcriptomics are contributing to unveiling the genetic basis of many complex routes in secondary plant metabolism and new technologies (like metabolic engineering or combinatorial biosynthesis) have been developed to obtain new and improved secondary therapeutic metabolites. This would enable the production, at medium/large scale and under controlled and sustainable conditions, of large quantities of plant metabolites with adjuvant activities.

In parallel, *in vitro* and *in vitro* approaches are needed to isolate novel activities linked to peptides and proteins.

In the last two decades, the ability to manipulate plant genomes has opened the door for manufacturing subunit vaccines from plants. Although many of the most promising new adjuvant formulations contain plant-made products among the key active components, the intrinsic capacity of the plant cell to generate a vast amount of partner molecules for cell interaction has somehow been neglected. Therefore, plant-based manufacturing platforms are ideal biofactories for the synthesis of the immunomodulators of the future, at least those of plant origin. The future plant-made vaccines may well go hand-to-hand with the discovery and recombinant biosynthesis of new adjuvant molecules (Granell *et al.* 2010).

While the continued use of plant-derived medicines in countries such as China and India, where traditional and modern medicine are integrated, may provide an opportunity to identify and isolate novel compounds using an ethnomedically guided approach and although plant medicines are used increasingly in Western society, there is still reluctance by the medical establishment to accept that these may have potential benefits to human health.

Thus, only the rigorous scientific investigation of plant-derived medicines will validate or refute any perceived or actual biological properties. In the case of immunostimulants and anti-cancers it will be fundamental to determine the mechanism of action by which plant extracts and their purified/recombinant active compounds (including secondary metabolites, proteins and peptides) exert their anti-cancer and adjuvant activity to allow their use in therapeutic vaccination.

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