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A natural polymer (NPX) as a new adjuvant for breast cancer vaccination strategies

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Summary

The use of proteins or peptides as immunogens is attractive for the development of vaccines, especially cancer vaccines, but requires efficient and safe adjuvant formulations to overcome their intrinsic weak immunogenicity. Although dozens of different adjuvants have been shown to be effective in preclinical and clinical studies, alum remains the only one approved for human use in the USA and the most employed worldwide, but it turned out to be inefficient in cancer vaccine formulations. Indeed, the prerequisites for an ideal cancer adjuvant differ from conventional adjuvants. Since cancer vaccines target self-antigens, the ideal cancer adjuvant must be extremely potent to circumvent immune tolerance, but it must also be safe to avoid autoimmune reactions. Of note, recent studies indicate that effective therapeutic and preventive cancer vaccines require the induction of a more balanced T helper 1 (Th1)/Th2 immune response, characterized by the presence of a strong cytotoxic $CD8^+$ T lymphocyte (CTL) activity, and the production of IgG subclasses with specific effector functions. For example, efficient tumor prevention in mice is associated with high levels of IgG2a and IgG2b subclasses, which are considered the most potent inducers of complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) in rodents. To solve all these problems, new generation vaccines often incorporate toll-like receptors (TLRs) agonists. Among them, natural polymers (NPs) that can act as damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs), are emerging as a new efficient class of vaccine adjuvants due to their ability to orchestrate the cross-talk between innate and adaptive immunity. In particular, NPs induce the maturation of dendritic cells (DCs) and finely regulate the balance between Th1 and Th2 responses, thus inducing potent and long-lasting humoral and cellular responses. Moreover, they are biocompatible, biodegradable, non-toxic, non-immunogenic, and non-inflammatory.

This project aimed at validating our NP, called NPX for patent constraints, as a new TLR agonist and carrier of immunogens for the design of more efficient and safer cancer vaccines, and comparing its adjuvanticity with alum. To this aim, NPX was chemically linked to the extracellular domain (ECD) of the rat form of the epidermal growth factor receptor (rHER2/neu), or to short peptide sequences derived from the ECD of rHER2/neu, and the resulting bioconjugates were used for immunization of both BALB/c and BALB-neuT transgenic mice.

In this work, we demonstrated that NPX has an extremely satisfactory safety profile, as no local side effects were observed in vaccinated mice. Moreover, NPX induced strong antigen-specific

immune reactions very efficiently. In fact, even though both alum- and NPX-adjuvanted formulations induced high humoral immune responses against rHER2/neu, NPX-vaccinated BALB/c mice disclosed IgG titers that were about two-fold higher than those calculated for alum. Of note, only conjugation with NPX induced a detectable humoral response against rHER2/neu-derived peptides. Both adjuvants induced high production of different IgG subclasses, but despite similar IgG1 titers NPX-vaccinated mice disclosed also higher IgG2a and IgG2b levels. The strong elicitation of the three IgG subclasses and the production of both Th1 and Th2-type cytokines such as IL-12p70, IFN-γ, IL-2, IL-6, and IL-10, confirmed the ability of NPX to induce a balanced Th1/Th2 response. Moreover, the different quality and quantity of IgG subclasses, and their superior ability to recognize rHER2/neu in its native conformation likely reflected on the better functionality of NPX-induced antibodies in triggering complement-mediated specific lysis of rHER2/neu-positive cells. Interestingly, NPX not only induced humoral responses that persisted over time, but also selected mature B cell clones secreting antibodies with an improved ability to bind rHER2/neu in its native conformation and to mediate effector functions. The robust immune responses induced by NPX proved to be effective in both the prophylactic and therapeutic settings; indeed, NPX-adjuvanted vaccine formulation prevented and significantly delayed tumor growth in tumor challenged mice. Interestingly, antitumor responses seemed in part to be mediated by NPX ability to induce also CTL responses, which were detected only in NPX-vaccinated groups. Finally, while both alum- and NPX-adjuvanted vaccines proved to be successful in breaking tolerance against rHER2/neu in BALB-neuT transgenic mice, NPX-vaccinated mice displayed IgG titers that were two-fold higher than those observed with alum. Interestingly, in transgenic mice NPX vaccination resulted in a better Th1/Th2 balance than in BALB/c mice. However, only NPX-induced antibodies were able to recognize rHER2/neu in its native conformation. This likely explains the capacity of NPX-based vaccination to protect from or delay the growth of spontaneous tumors in BALB-neuT mice, whereas alum completely failed to induce any protective response.

Taken together, our data show that NPX is a safe and powerful adjuvant that could be exploited for the development of new HER2/neu vaccination strategies. In fact, NPX is effective in enhancing the magnitude, breadth, quality, and longevity of specific humoral and cellular immune responses to antigens, without causing toxicity. Importantly, these effects can be achieved even with a strongly reduced antigen dose.

Riassunto

L'utilizzo di proteine o peptidi come immunogeni ha sempre rappresentato un'attrattiva per la creazione di vaccini, in particolare per i vaccini a scopo antitumorale; ciononostante, questo genere di formulazione richiede l'impiego di adiuvanti immunologici efficienti e sicuri che siano in grado di potenziare la scarsa immunogenicità degli antigeni stessi. Sebbene dozzine di adiuvanti si siano dimostrate efficienti in ambito preclinico e clinico, l'allume rimane il principale adiuvante ad uso umano impiegato in tutto il mondo, nonostante abbia dimostrato scarsa efficienza nell'ambito della vaccinazione antitumorale. I prerequisiti di un adiuvante antitumorale sono di fatto diversi da quelli dei classici adiuvanti. I vaccini antitumorali hanno infatti come target antigeni *self*, e dunque l'adiuvante antitumorale deve essere abbastanza potente da superarne la tolleranza immunologica, ma deve anche essere sicuro in modo da evitare fenomeni avversi di tipo autoimmune. Studi recenti hanno dimostrato che affinché i vaccini tumorali preventivi e terapeutici siano efficaci, si devono indurre risposte sia di tipo T helper 1 (Th1) che Th2, in grado di indurre sia una forte attività litica da parte dei linfociti $CD8^+$ T citotossici (CTL) che la produzione di sottoclassi anticorpali capaci di mediare particolari funzioni effettrici. Ad esempio, nel topo è stata dimostrata una correlazione tra prevenzione tumorale e presenza di alti livelli di immunoglobuline IgG2a e IgG2b, considerate le più efficienti nell'induzione di citotossicità complemento-dipendente (CDC) e di citotossicità cellulo-mediata anticorpo-dipendente (ADCC). Per adempiere a tutte queste richieste, le nuove generazioni di vaccini spesso incorporano nella propria formulazione degli agonisti dei *toll-like receptors* (TLRs). Tra questi agonisti, i polimeri naturali (NP), che agiscono come *damage-associated molecular patterns* (DAMPs) o *pathogen-associated molecular patterns* (PAMPs), stanno emergendo come una nuova classe di efficienti adiuvanti immunologici grazie alla loro capacità di mediare l'interazione tra il sistema immunitario innato e adattativo. In particolare, essi sono in grado di indurre la maturazione delle cellule dendritiche (DCs) e di regolare accuratamente il bilancio tra le risposte di tipo Th1 e Th2, al fine di indurre potenti e durature risposte umorali e cellulari. Gli NP sono inoltre biocompatibili, biodegradabili, non tossici, non immunogenici e non infiammatori.

Questo progetto di ricerca si è focalizzato sulla validazione del polimero naturale da noi sviluppato, cui ci riferiamo col termine NPX a causa di vincoli brevettuali, come nuovo agonista di TLR e veicolo di immunogeni per la creazione di vaccini antitumorali più efficienti e sicuri, comparando il suo profilo di adiuvanticità con quello dell'allume. A questo scopo, abbiamo coniugato NPX con il dominio extracellulare (ECD) del recettore 2 per il fattore di crescita

epidermico di ratto (rHER2/neu) o con peptidi a breve sequenza amminoacidica derivati dall'ECD stesso. I bioconiugati così sintetizzati sono stati utilizzati per la vaccinazione di topi BALB/c e di topi transgenici BALB-neuT.

In questo lavoro abbiamo innanzitutto dimostrato che NPX possiede un profilo d'azione estremamente sicuro, in quanto i topi immunizzati con tale adiuvante non hanno manifestato segni di tossicità a livello locale. NPX si è inoltre rivelato estremamente efficiente nell'indurre forti risposte immunitarie antigene-specifiche. Infatti, sebbene entrambi gli adiuvanti abbiano stimolato con successo elevate risposte umorali contro rHER2/neu, i topi BALB/c immunizzati con NPX hanno prodotto titoli di IgG doppi rispetto a quelli riscontrati nel gruppo trattato con l'allume. Inoltre solo NPX è riuscito a indurre la produzione di anticorpi in risposta alla vaccinazione con peptidi derivanti dalla porzione extracellulare del recettore. Entrambi gli adiuvanti hanno prodotto alti livelli delle differenti sottoclassi di anticorpi IgG; tuttavia, nonostante la produzione di IgG1 sia paragonabile, i livelli di IgG2a e IgG2b sono risultati nettamente maggiori negli animali vaccinati con NPX. Le elevate concentrazioni delle tre sottoclassi anticorpali e la produzione di citochine sia di tipo Th1, quali IL-12p70, IFN-γ e IL-2, che di tipo Th2, come IL-6 e IL-10, hanno confermato la capacità di NPX di indurre una risposta Th1/Th2 bilanciata. La differente qualità e quantità degli anticorpi prodotti e la loro migliore capacità di riconoscere il recettore nella sua conformazione nativa, probabilmente si riflettono nella migliore funzionalità degli anticorpi indotti da NPX nell'attivare la lisi cellulare rHER2/neu-specifica mediata dal complemento. In particolare, NPX si è dimostrato efficace non solo nell'indurre risposte umorali durature, ma anche nel selezionare cloni maturi di linfociti B secernenti anticorpi dotati di una migliore capacità di riconoscere il recettore nella sua forma nativa e di mediare le proprie funzioni effettrici. Le elevate risposte immunitarie indotte da NPX si sono dimostrate efficienti nell'ambito sia della vaccinazione preventiva che terapeutica, prevenendo o ritardando in entrambi i contesti la crescita tumorale nei topi inoculati con il tumore. Tali risposte sembrano inoltre essere almeno in parte dovute all'azione dei CTL, la cui presenza è stata riscontrata solamente negli animali immunizzati con NPX. Sia la vaccinazione con NPX che quella con l'allume si sono dimostrate in grado di rompere la tolleranza contro il recettore espresso costitutivamente nei topi transgenici BALB-neuT. Il gruppo vaccinato con NPX ha prodotto titoli anticorpali doppi rispetto al gruppo allume e un profilo di risposta Th1/Th2 ancora più bilanciato rispetto a quanto osservato nei topi BALB/c. Comunque, solo gli anticorpi indotti da NPX si sono dimostrati capaci di riconoscere il recettore nella sua conformazione nativa, e ciò spiegherebbe come questo tipo di vaccinazione riesca a conferire protezione o

indurre ritardo nella crescita tumorale spontanea nei topi BALB-neuT. Al contrario, la vaccinazione con l'allume ha completamente fallito nell'indurre una qualsiasi risposta tumorale protettiva.

Nell'insieme, i risultati ottenuti indicano che NPX è un adiuvante sicuro ed efficace, potenzialmente utilizzabile per la creazione di vaccinazioni antitumorali HER2-specifiche. Abbiamo infatti dimostrato che NPX migliora l'entità, l'ampiezza, la qualità e la longevità delle risposte immunitarie umorali e cellulo-mediate rispetto all'allume, senza causare effetti tossici e permettendo inoltre di utilizzare dosi ridotte di antigene.

Introduction

1. Cancer immunoediting: from immunosurveillance to tumor escape

In 1909 Paul Ehrlich first proposed the idea that nascent transformed cells arise continuously in our bodies, and that the immune system scans for and eradicates these cells before they are manifested clinically, thus hypothesizing that the immune system could repress a potentially "overwhelming frequency" of carcinomas (Ehrlich, 1909). However, this idea was not pursued until the mid-50s. In those years, different works involving the use of outbred strains of mice demonstrated that transplantable tumor recognition and elimination was mediated by allograft rejection and not tumor specific-rejection mechanisms, thus challenging the idea of the existence of tumor antigens (Dunn, Bruce *et al*., 2002). Subsequently, the availability of inbred strains of mice led to the demonstration that mice could be immunized against syngeneic transplants of tumors induced by chemical carcinogens or viruses, thus proving the existence of tumor antigens (Old and Boyse, 1964; Klein, 1966). These advances provided the foundation upon which Burnet and Thomas built their "cancer immunosurveillance" theory, a concept that formally envisaged that adaptive immunity can be responsible for preventing cancer development in immunocompetent hosts (Burnet, 1957; Thomas, 1959). As a consequence, hosts with impaired immune systems would exhibit increased incidences of spontaneous or chemically-induced tumors. To test this hypothesis, early experimental approaches comprehended neonatal thymectomy, heterologous anti-lymphocyte serum administration, or pharmacologic approaches as methods to obtain immunosuppressed mouse models. However, results obtained were highly discordant and gave little support for Burnet and Thomas' hypothesis (Burstein and Law, 1971; Stutman, 1975). Of particular note, some experiments showed that the cancer susceptibility of immunocompetent mice was similar to that of nude mice, which are now known to have major but not total immunologic defects (Stutman, 1974; Stutman 1979). Based on the limited understanding of these defects in the nude mouse available at that time, these results were highly convincing, and thus led to the abandonment of the immunosurveillance hypothesis.

By the 1990s, the availability of improved immunodeficient mouse models on pure genetic backgrounds allowed the reassessment of the role of immunity in cancer control. Interest in cancer immunosurveillance was rekindled by two key discoveries. The first one was the importance of endogenously produced interferon-γ (IFN-γ) in protecting the host against the growth of transplanted tumors and the formation of primary chemically-induced and

spontaneous tumors (Dighe, Richards *et al.*, 1994). Moreover, mice lacking either the IFN-γ receptor or the transcription factor that mediates IFN-γ receptor signaling, namely the signal transducer and activator of transcription 1 (STAT-1), were more susceptible to both carcinogen-induced and spontaneous primary tumor formation (Kaplan, Shankaran *et al.*, 1998). In fact, this cytokine promotes antitumor effector functions by stimulating the generation of $CD4^+$ Th1 lymphocytes and CTL, by activating macrophages, and by inducing major histocompatibility complex (MHC) class I expression on tumor cells (Bach, Aguet *et al.*, 1997). The second finding was the observation that mice lacking perforin were more prone to chemically-induced tumor formation, when compared to their wild-type counterparts (van den Broek, Kägi *et al.*, 1996; Street, Cretney *et al.*, 2001). Perforin is a component of the cytolytic granules of CTL and natural killer (NK) cells, and mediates lymphocyte-dependent killing of many different targets, including tumor cells (Russel and Ley, 2002). The existence of a cancer immunosurveillance process that is dependent on both IFN-γ and lymphocytes was finally supported using mice lacking the recombination activating gene 1 (RAG-1) or RAG-2. These genes are normally expressed in the lymphoid compartment and codify for enzymes involved in T-cell (TCR) and B-cell (BCR) receptor recombination, and in the repair of double-stranded DNA breaks. Mice deficient for either of these genes fail to rearrange lymphocyte antigen receptors and thus completely lack T and B cells, being more susceptible to carcinogen-induced or spontaneous tumor formation (Shinkai, Rathbun *et al.*, 1992; Shankaran, Ikeda *et al.*, 2001).

The immunosurveillance hypothesis also found evidences in humans. First, a correlation between the quantity and quality of tumor infiltrating lymphocytes (TILs) and a better prognosis was reported for a broad range of tumors, such as colon cancer (Naito, Saito *et al.*, 1998), esophageal cancer (Yasunaga, Tabira *et al.*, 2000), oral squamous cell carcinoma (Reichert, Day *et al.*, 1998), breast cancer (Yoshimoto, Sakamoto *et al.*, 1993), ovarian cancer (Sato, Olson *et al.*, 2005), and malignant melanoma (Haanen, Baars *et al.*, 2006). Of note, in all these studies the presence and location of $CD8^+$ TIL within the tumor and not at the tumor margin, appeared to have an important influence in the clinical outcome. Another evidence for cancer immunosurveillance is the observation that tumors of both viral and non-viral etiology are likely to develop in individuals with severe deficits of immunity (e.g., immunosuppressive therapies, primary immunodeficiencies), a feature that provided a strong argument to support the hypothesis that adaptive immunity can prevent tumor occurrence (Penn, 1999; Boshoff and Weiss, 2002). Finally, a major advance to the field of tumor immunology came from the demonstration that cancer patients can develop high levels of antibody and T cell responses to antigens expressed by their

tumors (Dougan and Dranoff, 2009). However, these immune responses can be observed even in patients with progressive diseases, indicating that immune recognition of cancer does not always result in immune protection.

Indeed, it has long been proposed that during tumor formation the immune system plays a dual role since it not only eliminates tumor cells, but also selects tumor variants that can better survive in an immunologically intact environment, as it happens with viruses, bacteria, and parasites (Dunn, Bruce *et al*., 2002). Experiments using tumors isolated from wild-type or RAG-2$^{-/-}$ mice and transplanted into RAG-2$^{-/-}$ recipients, have shown that cancers formed in the absence of an intact immune system are more immunogenic than tumors arising in immunocompetent hosts (Shankaran, Ikeda *et al.*, 2001). Moreover, lymphomas derived from perforin$^{-/-}$ mice grew rapidly when transplanted into perforin$^{-/-}$ mice, but were rejected when transplanted into wild-type mice (Street, Trapani *et al*., 2002). Taken together, these and other results showed that tumors are "sculpted" by the immunologic environment in which they form. This imprinting process can often result in the generation of tumor variants that are able to withstand the tumor-suppressing actions of the immune system, such as variants of reduced immunogenicity or that have acquired other mechanisms to evade or suppress immune attack. The alterations that occur during the immunologic sculpting of a developing tumor are probably facilitated by the inherent genetic instability of tumors; this selection particularly involves genes encoding tumor antigens, components of the antigen processing and presentation machinery, or components of the IFN-γ receptor signaling pathway (Dunn, Bruce *et al*., 2002).

The notion that the immune system not only protects the host against tumor formation but also shapes tumor immunogenicity is the basis of the "cancer immunoediting" hypothesis, which stresses the dual host-protective and tumor-promoting actions of innate and adaptive immunity on developing tumors (Dunn, Bruce *et al*., 2002; Dunn, Old *et al.*, 2004). Cancer immunoediting comprises three phases: elimination, equilibrium, and escape (**Fig. 1**).

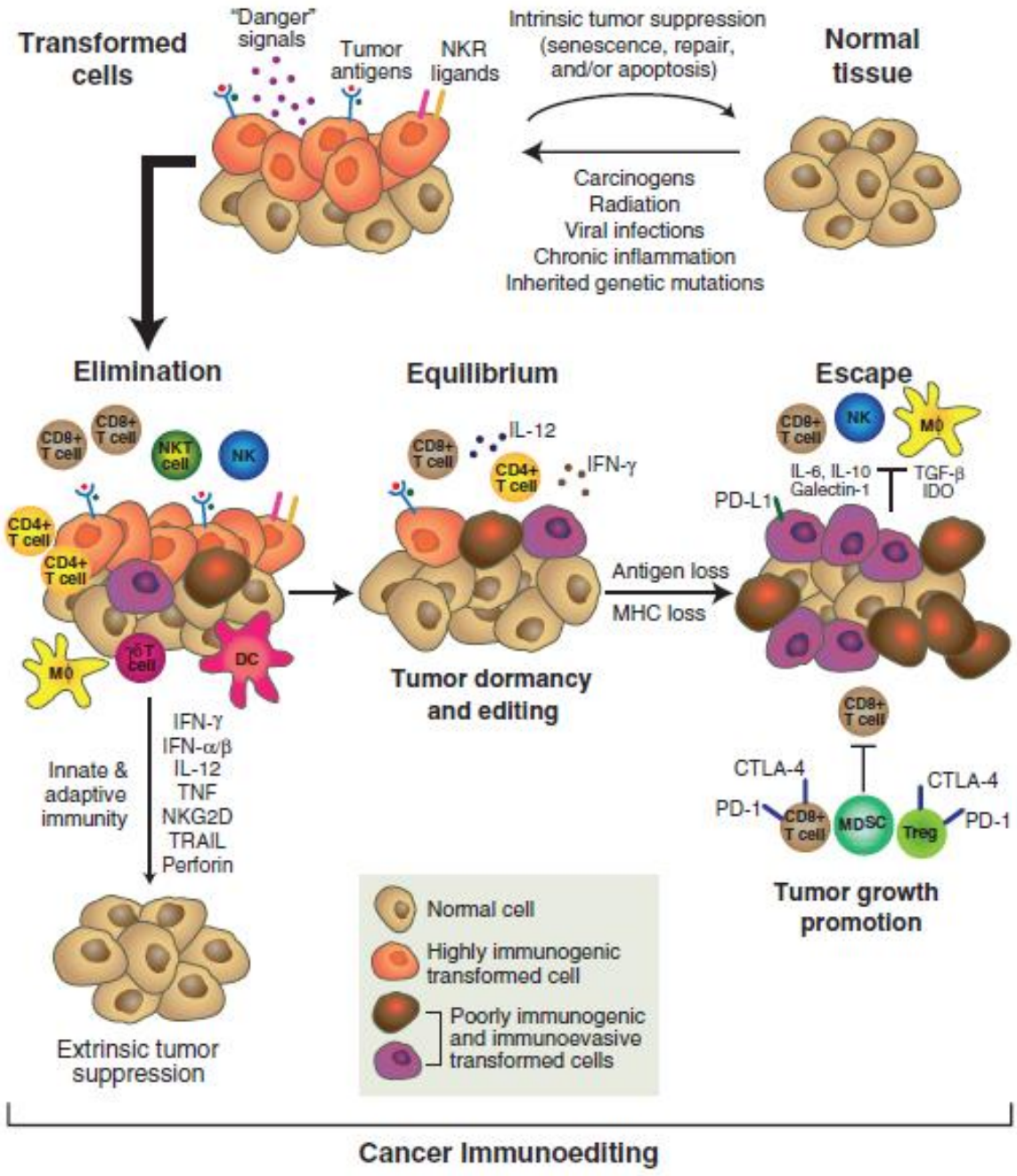


Figure 1. The cancer immunoediting concept. Cancer immunoediting is an extrinsic tumor suppressor mechanism that engages only after cellular transformation has occurred and intrinsic tumor suppressor mechanisms have failed. It consists of three sequential phases: elimination, equilibrium, and escape (from Schreiber, Old *et al.*, 2011).

The elimination phase is an updated version of cancer immunosurveillance, in which the innate and adaptive immune systems work together to detect the presence of a developing tumor and destroy it before it becomes clinically apparent (Dunn, Old *et al.*, 2004; Schreiber, Old *et al.*,

2011). The mechanism by which the immune system senses the presence of a developing tumor is not fully understood, but it seems in part due to danger signals such as type I IFNs, (Matzinger, 1994), or to the local stromal remodeling during tumor development. This process leads to the release of different DAMPs from dying tumor cells, such as high motility group box 1 (HMGB1), or from extracellular matrix (ECM) derivatives, such as hyaluronan fragments (fHA) (Shi, Evans *et al.*, Nature 2003; Powell and Horton, 2005).

If not all tumor cells are finally destroyed, rare surviving tumor cell variants may enter the equilibrium phase. During equilibrium, the adaptive immune system prevents tumor cell outgrowth and sculpts the immunogenicity of cancer cells, maintaining them in a functional state of dormancy. This phase depends on adaptive immunity only, and specifically on interleukin 12 (IL-12), IFN-γ, $CD4^+$ and $CD8^+$ T cells (Schreiber, Old *et al.*, 2011); moreover, it is the longest of the three phases, sometimes occurring over a period of many years in humans (Dunn, Old *et al.*, 2004).

In the escape phase, tumor cells that have acquired the ability to circumvent immune recognition and/or destruction emerge as progressively growing, visible tumors. Tumor cell escape can occur through different mechanisms, and likely reflects both reversible (e.g., epigenetic) and permanent (e.g. mutations and irreversible epigenetic) events.

As summarized in **Table 1**, the principal tumor immunoescape mechanisms are represented by:

a) loss of tumor antigen expression, through downregulation or mutation of the antigen itself, of MHC molecules, or of other antigen-processing molecules. These alterations impair the ability of tumor cells to efficiently present antigen-derived peptide to TILs, thus preventing recognition by CTL (Schreiber, Old *et al.*, 2011);
b) downregulation of costimulatory molecules in case of many hematopoietic malignancies, such as B7 family members, which induces a state of antigen-dependent anergy in responding effector cells (Rousseau, Hirschmann-Jax *et al.*, 2001);
c) secretion of immune inhibitors by tumor microenvironment or by tumor cells themselves, such as transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), interferons, prostaglandins, IL-10, and IL-6. VEGF plays a key role in recruiting immature myeloid cells from the bone marrow to the microenvironment, such as tumor-associated immature dendritic cells (TiDCs) and macrophages (TAMs) (Bellamy, Richter *et al*., 2001). Accumulation of TiDCs may cause DCs and T cells suppression through activation of indoleamine 2,3-dioxygenase (IDO). TGF-β and IL-10 can induce immunosuppressive regulatory DCs (DCregs) and T cells (Tregs). The latter are $CD4^+CD25^{high}FOXP3^+$ T lymphocytes that inhibit the function of tumor-specific T cells by producing

immunosuppressive cytokines, express the negative costimulatory molecules cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death 1 (PD-1) and its ligand (PD-L1), and consume IL-2, a cytokine critical for the maintenance of CTL functions (Schreiber, Old *et al.*, 2011). Other regulatory cells induced by proinflammatory cytokines are myeloid-derived suppressor cells (MDSCs), which are heterogeneous populations of immature myeloid cells showing abilities to suppress T cell function, and identified in mice as $Gr1^{+}CD11b^{+}$ cells. T cell dysfunction induced by MDSCs is probably mediated by STAT-3 and nuclear factor-κB (NF-κB) (Yaguchi, Sumimoto *et al.*, 2011). Of note, breast carcinoma can express mucins, such as DF3/MUC1, which inhibit the growth of activated T cells (Gimmi, Morrison *et al.*, 1996). Some colon carcinomas overexpress DcR3, a decoy receptor that neutralizes Fas ligand (FasL) which is used by CTL and NK to induce apoptosis on target cells (Pitti, Marsters *et al.*, 1998);

d) qualitative or quantitative alterations in components of T-cell signaling pathways, such as in the ζ chain of TCR, in $p56^{lck}$ and $p59^{fyn}$ proteins, in zeta-chain-associated protein kinase 70 (ZAP-70), and in the expression and translocation of NF-κB, all features that globally diminish T-cell activation (Rousseau, Hirschmann-Jax *et al.*, 2001; Kim, Emi *et al.*, 2007);

e) direct resistance to immune effector mechanisms by mutations in genes involved in triggering the apoptosis pathway (Müschen, Warskulat *et al.*, 2000).

Defects in antigen presentation	Adhesion deficiency
	MHC molecules/pathway defects
	Defects in antigen processing/transport
	Defects in costimulatory pathways
	Antigenic variants
	Decoy receptors
Microenvironment abnormalities	Inhibitory cytokines/ligands
	Growth/survival factors, angiogenesis
	Latency-associated proteins encoded by oncogenic viruses such as Epstein-Barr virus (EBV), human papillomavirus (HPV), human T-cell lymphotropic virus (HTLV), which induces tumor

	cell growth, angiogenesis and inhibit anti-tumor immune response
T-cell defects	Absence/deletion of specific T-cell precursors
	Anergy
	Downregulation of TCR ζ chain
	Mutation in signaling pathways
	Deletion/defect in helper T cell
	Defect in establishment of T-cell memory
	T-cell inhibitors

Table 1. Mechanisms by which tumors can escape immune surveillance (adapted from Rousseau, Hirschmann-Jax *et al.*, 2001).

2. Tumor antigens and cancer immunotherapy

The activation of the immune system against cancer has always been a major goal in immunology and oncology. Indeed, tumors express different types of proteins, called tumor antigens, which can be recognized by the immune system. Based on their pattern of expression, tumor antigens can be broadly divided into two categories: tumor-specific antigens (TSA), which are expressed only in tumor cells, and tumor-associated antigens (TAA), which instead can be expressed in tumor and normal cells.

TSA are relatively rare and comprise two distinct groups:

a) antigens derived from point mutations in oncogenes or tumor suppressor genes, such as *p53* which is detectable in a wide range of tumors such as breast (Miller, Smeds *et al*., 2005), lung (Ahrendt, Hu *et al*., 2003), and colorectal cancers (Rodrigues, Rowan *et al*., 1990), or *ras* in pancreatic, lung, and colorectal cancers (Minamoto, Mai *et al*., 2000);

b) proteins derived from virally-induced tumors, such as the human papillomavirus oncoproteins E6 and E7 (Duensing, Lee *et al.*, 2000) that cause cervical carcinoma, or latency-associated proteins encoded by EBV (Murray, Kurilla *et al*., 1992), which is associated with post-transplant lymphoproliferative disease (PTLD), Burkitt lymphoma, non-Hodgkin lymphoma and nasopharyngeal carcinoma. Among latent proteins, EBV-encoded nuclear antigen 1 (EBNA1) is

now emerging as a new promising target antigen, as recently demonstrated in a clinical trial (Icheva, Kayser *et al*., 2013), after being neglected for a long time due to impaired MHC-I processing and presentation.

TAA are more common antigens and can be grouped into four categories:

a) antigens belonging to the cancer/testis family, which comprises genes normally expressed by male germ cells, but also reactivated in malignant cells. Examples of these antigens include the melanoma antigen (MAGE), B antigen (BAGE), and G antigen (GAGE) families (Dalerba, Frascella *et al*., 2001; Scanlan, Gure *et al*., 2002);

b) antigens that are generally expressed during fetal development, but also reactivated in some tumors. For example, carcinoembryonic antigen (CEA), an oncofetal glycoprotein, is expressed in normal mucosal cells and overexpressed in adenocarcinoma, especially colorectal cancer, but also in other malignancies such as breast, lung, melanoma, bladder, and head and neck cancers (Hammaström, 1999). Alpha-fetoprotein (AFP) is a major fetal serum globulin found also in hepatocellular carcinoma (Soresi, Magliarisi *et al*., 2003) and more rarely in germ cell tumors of ovary (Maida, Kyo *et al*., 1998);

c) altered forms of surface glycolipids and glycoproteins, such as gangliosides, blood group antigens, and mucins that are overexpressed in particular in melanoma, ovarian, and breast carcinomas. This class of TAA is a target for cancer therapy with specific antibodies (Abbas, Lichman *et al.*, 2011);

d) unmutated self-proteins expressed at abnormally high levels. This group is very numerous and heterogeneous (see the database of the Academy of Cancer Immunology, http://cancerimmunity.org/peptide/overexpressed/). One of the best known example of this class of antigens is the human epidermal growth factor receptor 2 (HER2), which is overexpressed in breast, ovary, lung, pancreatic, prostate, and colon cancers (Srinivasan and Wolchok, 2004).

The discovery and characterization of tumor antigens suggest that even though cancer cells are less immunogenic than pathogens, the immune system is capable of recognizing and eliminating tumor cells. However, as described by the cancer immunoediting hypothesis, tumors can easily interfere with the development and function of the immune responses. Thus, the challenge for cancer immunotherapy is to develop strategies that can effectively and safely augment antitumor responses and circumvent the escape mechanisms triggered by tumor cells and tumor microenvironment. Taken together, anticancer immune strategies comprise both passive and active immunotherapy.

2.1 Passive immunotherapy

Passive immunotherapy generally does not stimulate the patient's immune system and bypasses the necessity of activating endogenous immunity. Anticancer passive immunotherapy consists in administration of immune stimulating cytokines, monoclonal antibodies (mAbs), and/or T cells (Wayteck, Breckpot *et al*., 2013).

Among immunostimulating cytokines, one of the most employed is IL-2, a T cell growth factor that promotes activation, proliferation, survival, and effector functions of anti-tumor T cells. It is approved by Food and Drug Administration (FDA) as it has demonstrated to be effective in metastatic renal cancer and melanoma, with complete cancer regression in 8% of the tested patients (Rosenberg, Yang *et al*., 1998). Another group of cytokines approved as first line or adjuvant therapy for many types of cancer is the IFN family. IFNα-2b, a highly pleiotropic cytokine with immunomodulatory, antiproliferative, differentiation-inducing, apoptotic, and antiangiogenetic properties, is commonly used for the treatment of renal and kidney carcinoma, follicular lymphoma, hairy cell leukemia, and chronic myelogenous leukemia (Baxevanis, Perez *et al*., 2009). Despite the effectiveness of cytokines in anticancer therapeutic regimens, their side effects are severe and often dose-limiting, due to non-specific stimulation and modulation of the immune system. The common cytokine-induced symptoms mirror those of systemic infection, such as hypotension, vomiting, diarrhea, fever, and malaise (Dougan and Dranoff, 2009).

Administration of mAbs has been employed with success in the clinic, thanks to their high specificity and long half-lives. Moreover, mAbs therapies are typically less toxic than conventional cytotoxic cancer chemotherapy, even if binding to nonmalignant cells can sometimes lead to significant adverse reactions (Hansel, Kropshofer *et al*., 2010). MAbs act through different mechanisms of action, in particular ADCC and CDC. ADCC involves the destruction of the antibody-coated cell by recruitment of effector cells such as NK, macrophages, and neutrophils, whereas CDC involves the activation of the complement cascade. Rituximab and ofatumumab, two CD20-specific mAbs approved for the treatment of hematological malignancies, mainly act through ADCC, while their activity is also partially dependent on CDC. By contrast, alemtuzumab, an anti-CD52 mAb, induces only CDC (Pillay, Gan *et al*., 2011). Other mAbs exert antitumor activities by blocking ligand binding to growth factor receptors, thus inhibiting signaling through these receptors. This leads to inhibition of proliferation, induction of apoptosis, and/or sensitization of cancer cells to chemotherapeutic agents. This is the case of cetuximab and panitumumab, two anti-epidermal growth factor receptor (EGFR) mAbs, or trastuzumab, an anti-

HER2 mAb. Other mAbs at present in clinical development are agonists of death receptors. Mapatumumab is an agonistic human mAb that binds to the tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and directly induces the death of cancer cells through apoptosis (Adams and Weiner, 2005). Other molecules display immunomodulating properties, like the FDA-approved mAb Ipilimumab, which targets CTLA-4 on the surface of Tregs and thus inhibits the development of peripheral immune tolerance. Similarly, tremelimumab has been tested in several phase I–II clinical studies to evaluate its pharmacological profile in metastatic melanoma and metastatic renal cell carcinoma (Ribatti, 2014). Finally, mAbs can be conjugated to radioisotopes, toxins, cytokines, enzymes or cytotoxic drugs to improve their delivery to tumor cells while reducing the side effects associated with systemic administration. Y-90 ibritumomab tiuxetan and I-131 tositumomab, two radiolabeled mAbs specific for CD20 antigen, are approved for the treatment of non-Hodgkin lymphoma, (Pillay, Gan *et al*., 2011). Gemtuzumab ozogamicin is a calicheamicin immunoconjugate targeting CD33 approved for the treatment of acute myeloid leukaemia (AML), but voluntarily withdrawn after a post-marketing phase III trial in 2010 because no real improvement in clinical benefit was observed (http://www.fda.gov/safety/medwatch/safetyinformation/safetyalertsforhumanmedicalproducts/ucm216458.htm). **Table 2** reports the main mAbs presently approved in oncology.

Antibody	Target	FDA-approved indication	Main mechanism of action
Trastuzumab (Herceptin)	HER2	(a) HER2-positive breast cancer, as a single agent or in combination with chemotherapy for adjuvant or palliative treatment (b) HER2-positive gastric or gastroesophageal junction carcinoma, as first-line treatment in combination with cisplatin and capecitabine/5-fluorouracil	Inhibition of HER2 signaling; ADCC
Bevacizumab (Avastin)	VEGF	Palliative treatment of colorectal cancer, non-squamous non-small cell lung cancer, glioblastoma, or renal cell carcinoma	Inhibition of VEGF signaling
Cetuximab (Erbitux)	EGFR	(a) Initial treatment of locally or regionally advanced squamous cell cancer of the head and neck (SCCHN) in combination with radiation	Inhibition of EGFR signaling;

		therapy (b) Single agent for SCCHN patients who failed prior platinum-based therapy (c) Palliative treatment of pre-treated metastatic EGFR-positive colorectal cancer	ADCC
Panitumumab (Vectibix)	EGFR	Single palliative treatment of pre-treated EGFR-expressing metastatic colorectal carcinoma	Inhibition of EGFR signaling
Ipilimumab (Yervoy)	CTLA-4	Treatment of unresectable or metastatic melanoma	Inhibition of CTLA-4 signaling
Rituximab (Rituxan® and Mabthera)	CD20	(a) Treatment of CD20-positive B cell non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL) (b) Maintenance therapy for untreated follicular CD20-positive NHL	ADCC; direct induction of apoptosis; CDC
Ofatumumab (Arzerra)	CD20	Treatment of patients with CLL refractory to fludarabine and alemtuzumab	ADCC; CDC
^{90}Y-Ibritumomab Tiuxetan (Zevalin)	CD20	(a) Treatment of relapsed or refractory, low-grade, or follicular B cell NHL (b) Previously untreated follicular NHL in patients who achieve a partial or complete response to first-line chemotherapy	Delivery of the radio-isotope yttrium-90
^{131}I-Tositumumab (Bexxar)	CD20	Treatment of patients with CD20 antigen-expressing relapsed or refractory low-grade, follicular, or transformed NHL	Delivery of the radio-isotope iodine-131; ADCC; direct induction of apoptosis
Brentuximab vedotin (Adcetris)	CD30	Treatment of relapsed or refractory Hodgkin lymphoma and systemic anaplastic lymphoma	Delivery of toxic payload, auristatin toxin

Alemtuzumab (Campath)	CD52	Single agent for the treatment of B cell CLL	Direct induction of apoptosis; CDC

Table 2. Characteristics of currently FDA-approved mAbs in oncology (adapted from Scott, Allison *et al.*, 2012).

The treatment of cancer patients with T cell populations that have been previously expanded *ex vivo* is called adoptive T cell therapy (ACT). After re-infusion into the patient, T cells can traffic to the tumor and mediate its destruction (Restifo, Dudley *et al.*, 2012). Two major sources of T lymphocytes for ACT are TILs and the peripheral blood of the patient. T cell harvesting from peripheral blood is technically easier, but this approach proved to be effective only when the frequency of tumor antigen-specific T cell precursors is relatively high. This is the case of EBV-specific T cells, which can be easily expanded virtually from all EBV-positive subjects and have been infused in a relevant number of patients with virus-driven malignancies (Bollard, Rooney *et al.*, 2012). TILs from the tumor mass or its adjacent lymph nodes contain an even higher frequency of tumor-reactive cells (Dougan and Dranoff, 2009). After isolation, TILs are generally cultured *in vitro* with IL-2, and the populations displaying the desired TCR specificity are selected and expanded (Rosenberg, Packard *et al.*, 1988). Before re-infusion of T cells, patients are immunodepleted by chemotherapy alone or chemotherapy in combination with total-body irradiation. This non-myeloblative, lymphodepleting step is associated with enhanced persistence of the transferred T cells (Dudley, Wunderlich *et al.*, 2005; Gattinoni, Powell *et al.*, 2006). In spite of successful outcomes in metastatic melanoma patients, the protocol just described has its major limitation in the recruitment of sufficient numbers of TILs in other cancer histotypes than melanoma (Wayteck, Breckpot *et al.*, 2013). Moreover, isolation and expansion of TILs can take 5 to 6 weeks, making this treatment difficult for patients with progressive disease (Ito and Chang, 2013). In order to overcome these problems, T cells can be genetically engineered using three different approaches represented in **Fig. 2**. The first strategy consists in engineering patient T cells to express TCRs that have been selected for tumor recognition (**Fig. 2a**). Successful responses have been observed in metastatic melanoma; however, TCR recognition of antigen is MHC-restricted, and thus engineered TCR can only be used in patients with the appropriate MHC allele (Morgan, Dudley *et al.*, 2006). MHC restriction can be bypassed by engineering T cells to express novel chimeric antigen receptors (CARs) (**Fig. 2b**). CARs can be generated in a variety of ways. Most commonly, sequences encoding the variable regions of antibodies are engineered to

encode a single chain, which is genetically engrafted onto the TCR intracellular domains that are capable of activating T cells (Restifo, Dudley *et al.*, 2012). The main advantage of CARs is represented by the ability to redirect T lymphocyte activity towards a selected, even non protein, target antigen in a non-MHC-restricted fashion, exploiting the antigen-binding properties of mAbs. The recognition of tumor antigens in a non-MHC-restricted manner enables CAR-expressing cells to be not affected by down-regulation of human leukocyte antigen (HLA) class I antigens and by defects in the antigen-processing machinery of tumor cells (Baxevanis and Papamichail, 2004). Another approach is to isolate TCRs from humanized mice that have been primed to recognize tumor antigens (**Fig. 2c**). Mouse T cells specific for the MHC-restricted epitope of interest can be isolated, and their TCR genes cloned into recombinant vectors used to genetically engineer T cells collected from the patient (Restifo, Dudley *et al.*, 2012).

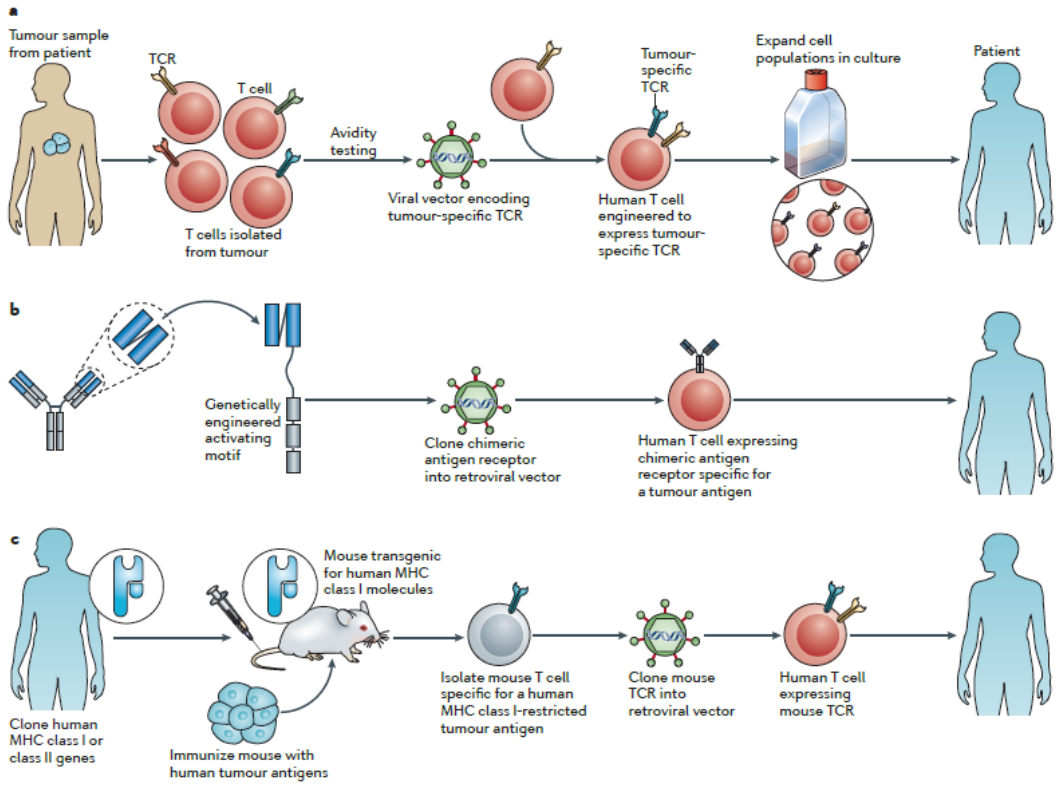


Figure 2. Approaches to confer specificity for tumor antigens by genetic engineering of T cells. (**a**) T cells are harvested from the tumor of the patient and their TCRs can be cloned and inserted into retroviruses or lentiviruses in order to infect autologous T cells. (**b**) Alternatively, T cells are engineered to express CAR, and thus problems concerning the MHC-restricted TCR recognition of a tumor antigen can be circumvented. (**c**) TCR can be also obtained from humanized mice previously immunized with the antigen of

interest. Mouse T cells specific for the MHC-restricted epitope are isolated, their TCR cloned and used to genetically engineer T cell from the patient (from Baxevanis, Perez *et al*., 2009).

2.2. Active immunotherapy: cancer vaccines

Active immunotherapy approaches, namely cancer vaccination, consist of stimulating the patient's own immune system to recognize and destroy malignant cells. The advantages of this strategy is the induction of a sustained anti-tumor response that can lead to immunological memory, thus insuring tumor protection against future relapses (Ito and Chang, 2013). Differently from active immunization for infectious disease, which triggers robust immune responses against foreign antigens, cancer vaccination has to deal with antigens perceived by the host's immune system as self-proteins. Hence, the major challenge for cancer vaccines is to break the immune tolerance to the TAA of interest, mounting an effective and specific anti-tumor immune reaction against malignant cells (Mocellin, Rossi *et al*., 2004; Strioga, Darinskas *et al.*, 2014). Current cancer vaccines attempt to improve tumor antigen presentation and host T lymphocyte activation by enhancing antigenic peptide-MHC molecule stability, by restoring costimulatory signals, and by amplifying recruitment of the patient's immune effector cells (Rousseau, Hirschmann-Jax *et al.*, 2001). To achieve these goals, several vaccine formulations have been designed, which can be divided into seven main groups (**Fig. 3**):

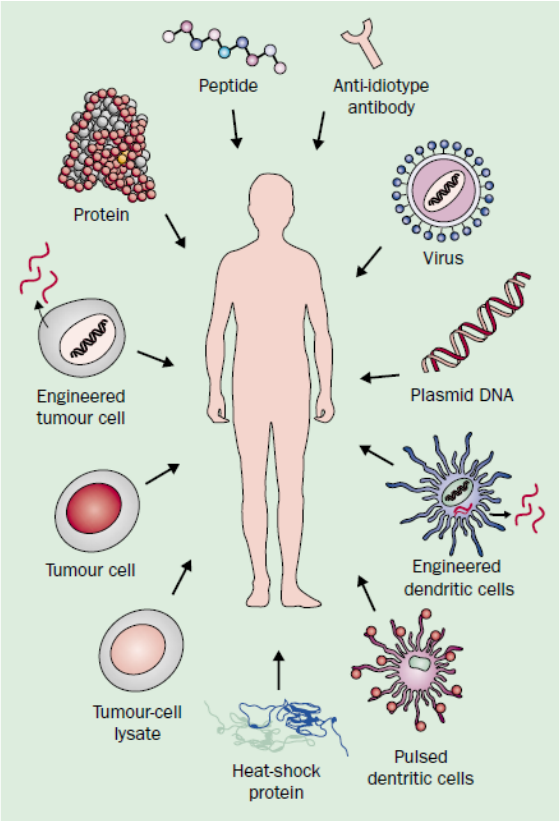


Figure 3. Principal approaches investigated for cancer vaccination (from Mocellin, Mandruzzato *et al.*, 2004).

a) **Whole tumor cells or tumor cell lysates vaccines.** The first types of antitumor vaccines were composed of either irradiated or lysated human autologous or allogenic tumor cells. Use of whole cells as antigen sources was appealing since these vaccines contain a large repertoire of tumor antigens and bypass the need for specific epitope identification (Mocellin, Mandruzzato *et al.*, 2004; Pejawar-Gaddy and Finn, 2008). Tumor cells can be also engineered to become more immunogenic, for example by inducing the expression of costimulatory molecules and/or cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-2, which recruit and activate antigen-presenting cells (APC) at the injection site, thus favoring the upload of tumor antigens and their presentation to T cells in secondary lymphoid organs (Pardoll, 1998; Mocellin, Mandruzzato *et al.*, 2004).

b) **DC-based vaccines.** Since DC are the most powerful activators of naïve T cells and play a crucial role in activating antigen-specific immune responses (Dougan and Dranoff, 2009; Lewis and Reizis, 2012), they are widely used in antitumor vaccination protocols. DCs can be directly isolated from peripheral blood or obtained through *ex vivo* culturing from peripheral blood precursors. In both cases, the cells are then loaded with tumor peptides, whole proteins, whole

necrotic or apoptotic tumor cells, tumor cell lysates and transfected or transduced with tumor-derived DNA or mRNA and viral vectors containing the tumor antigen of interest, respectively. Alternatively, DCs can be fused with tumor cells (Gilboa, 2007; Pejawar-Gaddy and Finn, 2008). DC-based cancer vaccines appear promising in terms of efficacy, but this approach is afflicted by two main problems emerged in clinical trials, i.e. the need to define a standardized protocol and to minimize cost and time required for such treatments. The latter problem was in part solved by the direct delivery of antigens to DCs *in vivo*; however, a better understanding of DC biology in tumor environment will provide a rationale for choosing the best DC population and modulation method for optimized DC-based vaccination protocols (Palucka, Ueno *et al*., 2011).

c) **DNA or RNA-based vaccines.** The use of naked DNA or RNA encoding the tumor antigen of interest as a vaccine has been extensively exploited because of their ease of production (Pejawar-Gaddy and Finn, 2008). Administration of DNA or RNA by injection or gene gun introduces tumor antigen genes directly into DCs for endogenous processing and presentation to T and B cells, or into other host cells for cross-presentation by DCs (Rice, Ottensmeier *et al*., 2008). This vaccination strategy seems to mediate antibody and cellular immune responses in mice; however, some preclinical experiments have shown engagement of Th2-skewed responses, leading to inefficient CTL responses (Berzofsky, Terabe *et al*., 2004; Nava-Parada, Forni *et al*., 2007). To partly circumvent this problem, naked DNA or RNA can also contain specific sequences encoding for molecules such as oligo- cytosine phosphoguanosine (CpG), which are able to potentiate and regulate immune responses, and thus called immune potentiator or immune adjuvants (Mocellin, Mandruzzato *et al*., 2004).

d) **Recombinant viral and bacterial vaccines.** Naked DNA and RNA can be made more immunogenic by incorporating them into viral or bacterial vectors. Both vectors are an attractive choice for antigen delivery because they mimic a natural infection, providing the necessary “danger signals” required for optimal activation of APCs (Pejawar-Gaddy and Finn, 2008). However, the possible preexisting immunity against viral vectors may attenuate antitumor responses (Saxena, Van *et al*., 2013) and the immunodominance of viral or bacterial antigens over tumor antigens can lead to weak responses against the latter ones (Berzofsky, Terabe *et al*., 2004).

e) **Anti-idiotypic antibody-based vaccines.** Administration of mouse mAbs specific for a tumor antigen induces the formation of autologous antibodies (Abs) against mAbs. Such Abs specific for determinants within the antigen binding site of the original mAb are called anti-idiotypic antibodies. The anti-idiotypic antibody could mimic the antigen that induced the original antibody, and thus it can function as a surrogate antigen able to stimulate specific immune

responses. The advantage of anti-idiotypic antibody vaccination is that it can confer protection against non-protein antigens, such as tumor specific sugars or lipids (Pejawar-Gaddy and Finn, 2008; Gómez and Ardigo, 2012).

f) **Heat-shock protein-based vaccines.** These ubiquitous intracellular proteins function as chaperones for peptides, including those derived from tumor antigens. All DCs express a specific receptor for heat-shock proteins, named CD91, whose engagement triggers immature DCs (iDCs) to become potent APC. Therefore, heat-shock proteins can be exploited as an endogenous signal and a vehicle for DCs to cross-present tumor antigens (Mocellin, Mandruzzato *et al*., 2004). The ability of heat-shock protein-peptide complexes to stimulate both innate and adaptive responses has recently shown promising results in patients with initial stages of melanoma and renal clear cell cancer (Ciocca, Cayado-Gutierrez *et al*., 2012).

g) **Recombinant proteins and antigenic peptides.** Protein- and peptide-based antigen vaccines were among the first defined vaccines demonstrating both protective and therapeutic efficacy in animal models (Lollini, Cavallo *et al*., 2013). As TAA and, even more, TAA-derived peptides are in general poorly immunogenic, this strategy is usually based on the administration of these products with an appropriate immunological adjuvant in order to optimize antitumor responses. Protein-based vaccines represent an appealing type of cancer vaccination as some recombinant proteins are easily producible by recombinant technology and circumvent the need to know the sequence of immunogenic epitopes (Mocellin, Mandruzzato *et al*., 2004). With regard to anti-HER2 positive breast cancer vaccination, it has been demonstrated that vaccination with whole HER2 protein, its intracellular domain (ICD) or ECD together with adjuvants or cytokines, displayed strong immune responses (Ladjemi, Jacot *et al*., 2010). In fact, proteins usually contain several MHC restricted epitopes recognized by both CTL and Th lymphocytes, and linear or conformational epitopes that can be recognized by Abs. However, for those recombinant proteins whose production is high cost and laborious, as regard also safety control (Lollini, Cavallo *et al*., 2013), the use of peptide as vaccines represents a valid alternative option. Indeed, peptides are easier to produce and seem to be more specific and able to elicit stronger humoral and cellular responses according to the MHC type restriction of the epitope identified (Ladjemi, Jacot *et al*., 2010). The first peptide vaccines investigated were composed of MHC class I-restricted epitopes, also defined as CTL epitopes, but immunological and clinical responses observed were limited. In fact, usually free peptides likely have poor pharmacokinetic properties, and due to a short half-life, they may be rapidly cleared before being loaded onto DCs. Without any adjuvant, DCs might not mature and promote tolerance (Mellman, Coukos *et al*., 2011). Several approaches have been

tested to overcome these problems. For example, as preclinical studies have demonstrated that tumor-specific Th cells critically contribute to the development and efficacy of antitumor responses, CTL epitopes physically linked to Th epitopes were explored with successful results (Sotiriadou, Kallinteris *et al*., 2007). Alternatively, CTL epitopes can be conjugated to TLRs ligands, or to universal, non-specific MHC class II-restricted epitopes, such as pan-DR Th epitope (PADRE) (Baxevanis, Perez *et al*., 2009; Rescigno, Avogadri *et al*., 2007). Finally, another promising strategy to potentiate both protein- and peptide-based vaccines is the use of DC-activating adjuvants, in particular TLR ligands such as monophosphoryl lipid A (MPL) or poly-L-lysine and carboxymethylcellulose (poly-ICLC, Hiltonol) (Cluff, 2010; Kimura, McKolanis *et al*., 2013).

All the approaches described above have been exploited in both therapeutic and prophylactic cancer vaccination strategies.

The vast majority of cancer vaccines in development are therapeutic cancer vaccines, which are designed for the administration to patients already diagnosed with cancer. In particular, because many tumors can be surgically removed and there is often a latency period between removal and tumor recurrence, cancer vaccines have been proposed as an approach to elicit and/or boost antitumor immunity in patients with minimal residual disease, thereby preventing relapse or prolonging the time to recurrence (Finn, 2003). However, the success rate of therapeutic cancer vaccines is not impressive (Rosenberg, Yang *et al*., 2004). In 2010 the only one of the more than 1000 therapeutic cancer vaccines tested in clinical trials that has been approved by FDA, named sipuleucel-T or Provenge (Dendreon) (Schreiber, Raez *et al*., 2010), had arose new interest in this therapeutic field. Provenge is approved for the treatment of patients with metastatic, castration-resistant prostate cancer (CRPC), as it is designed to stimulate T cell immune responses against prostate acid phosphatase (PAP), an antigen highly expressed by prostate cancer cells but not by other tissues. Provenge consists of autologous APCs loaded with PAP linked to the immunostimulatory cytokine GM-CSF (Yaddanapudi, Mitchell *et al*., 2013). Despite the initial clamor for Provenge, clinical monitoring showed the weak efficacy of this treatment. The failure is probably attributable to an incomplete understanding of the relationship between tumor development, tumor microenvironment and immune system. In particular, therapeutic vaccination must bypass immune regulatory mechanisms that have already led to tumor tolerance (Dougan and Dranoff, 2009). The inability of therapeutic vaccination to eradicate tumors may be due to both the intratumoral and systemic suppressive milieu orchestrated by the tumor (Baxevanis, Perez *et al*., 2009), the so called tumor micro- and macroenvironment (Gabrilovich, Ostrand-Rosenberg *et al*, 2012). Consequently, clinical trials are planned to combine

Provenge with anti-CTLA-4 or anti-PD-1 treatments in order to improve its efficacy. To date, the combination of vaccination with antibodies, cytokines, chemokines, enzymes, or ligands of TLRs that implement and modulate antitumor responses is a common strategy (Berzofsky, 2011); however, this "push-pull" approach should be better investigated to compare risks to benefits. Indeed, the use of anti-CTLA-4 or anti-PD-1 mAbs can sometimes cause significant toxicities, namely autoimmune reactions, due to lack of specificity. Moreover, an important point that should not be ignored is the high cost of these treatments, plus the cost of hospitalization due to their administration and/or increased toxicity. Hence, new immunological adjuvants and delivery systems are now under development as potentially cheaper, safer, and more efficient strategies to improve anticancer vaccination protocols (Finn, 2014).

Prophylactic vaccines have been used successfully for the prevention of cancers of viral etiology, such as hepatitis B virus (HBV) and HPV. In fact, HBV vaccines protect against HBV infection, and the potentially subsequent cirrhosis and hepatocellular carcinoma. The original anti-HBV vaccine, Heptavax (Merck), was approved in 1981, and was the first anticancer prophylactic vaccine to get into the clinic. The vaccine is based on nanoparticles containing the recombinant HBV surface antigen (HBsAg), which is highly immunogenic and able to convey lifelong immunity. The two FDA-approved HPV vaccines, Gardasil (Merck) and Cervarix (GlaxoSmithKline), disclose effective protection against cervical infection with the two main types of HPV (HPV type 16 and 18), which cause approximately 70% of all cases of cervical cancer worldwide (Yaddanapudi, Mitchell *et al*., 2013). Moreover, these vaccines have been shown to prevent also vaginal, vulvar, and anal precancerous lesions (Ito and Chang, 2013). Similar to other vaccines for the prophylaxis of viral infections, the efficacy of Heptavax, Gardasil, and Cervarix relies on the generation of a strong neutralizing antibody response against immunodominant viral antigens (Yaddanapudi, Mitchell *et al*., 2013). However, the major challenge in prophylactic vaccination remains the design and validation of vaccines that specifically target cancers not associated to infectious agents. The decisive support for preventive anticancer vaccines had come from many years of experiments in animal models, which thanks to genetic engineering progressively better recapitulate human diseases. These preclinical studies have highlighted the high effectiveness and safety profile of prophylactic vaccination against various types of cancer antigens (Finn and Forni, 2002; Iinuma, Homma *et al*., 2004; Garcia-Hernandez, Gray *et al*., 2008). Indeed, prophylactic vaccination can elicit tumor-specific immunity and establish also long-term memory without causing autoimmune reactions, as widely demonstrated in the case of breast cancer vaccines (Disis, Gooley *et al*., 2002; Tanaka, Amos *et al*., 2003).

3. Vaccine immunology: from innate to adaptive immunity

Long-term protection is a complex challenge for vaccine development. It requires the persistence of Abs and/or the generation of immune memory cells capable of rapid and effective reactivation upon subsequent antigen exposure. Parameters of immune memory induction, namely the presence of persisting Abs and immune memory cells, are thus essential to evaluate long-term vaccine efficacy (Plotkin, Orenstein *et al*., 2012).

Vaccine-induced immune effectors are essentially Abs, capable of specific binding to toxins, pathogens and others extracellular antigens, and cytotoxic $CD8^+$ T lymphocytes, which can limit the spread of diseases by recognizing and killing target cells directly or by secretion of specific cytokines (Pardoll, 2002; Plotkin, Orenstein *et al*., 2012). The generation and maintenance of both humoral and cellular responses are supported by growth factors and signals released by $CD4^+$ T helper lymphocytes. According to their specific effector mechanisms, they are grouped into different subsets, Th1 and Th2 being the most common ones. These cells are controlled by Treg that are involved in maintaining immune tolerance (Bacchetta, Gregori *et al*., 2005). The induction of antigen-specific B and T cell responses requires activation through specific APCs, essentially DCs, which must be recruited into the site of antigen administration.

Briefly, following vaccine injection, antigens and/or adjuvants must provide sufficient danger signals to attract to the site of injection cells of the innate immune system that trigger an inflammatory reaction (Lollini, Cavallo *et al*., 2006). Components of vaccine formulation of viral or bacterial origin trigger in general a classical inflammatory reaction; conversely, when the inflammation is not pathogen-induced, a reaction termed “sterile inflammation” arises. In both cases, the same host receptors mediate the immune responses, and have been collectively termed pattern recognition receptors (PRRs). These germline-encoded receptors are subdivided 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 that are characterized by the presence of a carbohydrate-binding domain; e) absence in melanoma 2 (AIM2)-like receptors, characterized by the presence of a pyrin domain and a DNA-binding HIN domain involved in the detection of intracellular microbial DNA. PRRs recognize conserved structural moieties that are found in microorganisms, named PAMPs, and also non-infectious material that can cause tissue damage and endogenous

molecules that are released during cellular injury or cell death, called DAMPs. DAMPs have similar functions as PAMPs in terms of ability to activate proinflammatory pathways. While PAMPs are able to trigger classical inflammatory reactions, DAMPs are involved in sterile inflammations. In both cases, following ligand recognition PRRs activate downstream signaling pathways, such as NF-κB, mitogen-activated protein kinase (MAPK), and type I interferon pathways, resulting in the upregulation of proinflammatory cytokines and chemokines (Chen and Nuñez, 2010). Among PRRs, TLRs play a central role in sensing danger signals; moreover, as TLR subtypes senses different stimuli, every TLR-ligand triggers the secretion of different molecules, thus influencing and regulating the type of adaptive immune responses elicited (Pulendran, 2004).

If an appropriate inflammatory microenvironment arises as a result of vaccine injection, extravasation and attraction of PRRs-expressing innate immune cells occur. In fact, these cells, such as iDCs, monocytes and neutrophils, recognize DAMPs or PAMPs from the microenvironment through their PRRs, become activated, and migrate along the lymphatic vessels to the draining lymph nodes where they in turn activate T and B lymphocytes (Plotkin, Orenstein *et al*., 2012). Among cells of the innate compartment, mature DCs (mDCs) play a crucial role in sensing foreign antigens, either directly or indirectly, and regulating the strength, quality, and persistence of the adaptive immune response. Thus, DCs are the bridge between innate and adaptive immunity (Pulendran and Ahmed, 2006; Schijns, Tartour *et al*., 2014). Antigen recognition, in particular through TLRs, triggers DCs maturation, which occurs during their migration toward the draining lymph nodes and is characterized by antigen processing and modification of the expression of homing receptors (Pardoll 2002). In fact, iDCs express low levels of MHC class I and II, costimulatory and adhesion molecules such as CD40, CD80 and CD86 and, hence, are poor stimulators of T cells. Upon recognition and internalization by DCs, exogenous antigens are degraded into small antigenic peptides in phagolysosomes and processed for presentation in the context of MHC-II molecules, while endogenous antigens are processed in proteasomes and presented in the context of MHC class I molecules. However, exogenous antigens can also be presented through MHC class I, and endogenous ones via MHC class II (Beverley, 2002). In the draining lymph nodes mDCs present antigenic peptides to naïve $CD4^+$ 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 is primarily regulated by signaling events derived from MHC-peptide and TCR interaction (signal 1), and from costimulatory and adhesion molecules such as CD80, CD86, CD40, CD54, CD58 on mDCs (or other APCs) and CD28, CD154, CD2, CD11a on T cells (signal 2). For example, CD80 and/or CD86-CD28 interactions provide important signals for T cell

activation and survival, while CD40-CD154 interactions are crucial for the development of $CD4^+$ T-dependent effector functions such as help for B cell differentiation and class switch. Additional signals are immunomodulatory cytokines (signal 3) produced by activated APCs that dictate the polarization of $CD4^+$ T cells towards Th1 or Th2 subtypes: IL-12 has an important role in the differentiation of Th1 cells, while IL-4 is crucial for Th2 differentiation (Aimanianda, Haensler *et al*., 2009). Notably, in the absence of appropriate danger signals, DCs remain immature: upon contact with naïve T cells, T cells do not differentiate into immune effectors, but into Treg that maintain immune tolerance (Bacchetta, Gregori *et al*., 2005). Immune responses and consequent disease outcome are influenced by many factors, but Th1 and Th2 lymphocytes represents the key elements in controlling the functions of other immune cells. Many cytokines are produced by both Th subtypes, such as tumor necrosis factor α (TNFα), IL-3, IL-6, and GM-CSF; however, only Th1 effectors produce IL-2 and 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 antibody-dependent mechanisms, principally ADCC and CDC. Thus, the balance between Th1 and Th2 responses is a critical step to be considered during vaccine formulation (Beverley, 2002).

In experimental animal models, numerous factors have been shown to influence the preferential differentiation of $CD4^+$ T cells towards Th1 or Th2 pathways. These determinants include:

a) dose of antigen, as lower vaccine doses are classically associated with preferential Th1 responses;
b) route of administration, which targets distinct populations of DCs. For example, skin DCs preferentially induce Th1 responses;
c) the extent and type of DCs activation, which is the main determinant of $CD4^+$ T cell differentiation (Plotkin, Orenstein *et al*., 2012; Beverley, 2002);
d) the type of vaccine adjuvant, as for example alum induces Th2-skewed immune responses (Lambrecht, Kool *et al*., 2009).

As regards B cells, these lymphocytes are activated in the lymph nodes or spleen that have been reached by vaccine antigens, upon diffusion and/or in association to migrating DCs. In fact, naïve B cells generated in the bone marrow circulate through the body until they encounter a protein antigen that their specific surface IgM receptor may bind. Antigen recognition triggers B cell activation and upregulation of C-C chemokine receptor type 7 (CCR7), that drives antigen-specific B cells towards the outer T cell zone of secondary lymphoid tissues where they are exposed to recently activated DCs and T cells (Förster, Davalos-Misslitz *et al*., 2008). These interactions provide B cell activating signals, which rapidly drive B cell differentiation into plasma cells

secreting low-affinity germline Abs, in what is called the extrafollicular reaction (MacLennan, Toellner *et al*., 2003). During this differentiation, immunoglobulin (Ig) class-switch recombination from IgM toward IgG, IgA, or IgE occurs, owing to $CD4^+$ Th1 and/or Th2 cells essential helper function, as this process is dictated by the engagement of CD40L molecules on Th cells with CD40 on B cells. While in humans the distinction is not completely clear, in rodents IFN-γ producing Th1 T cells promote class-switch towards IgG2a, whereas Th2 cells support the generation of IgG1 and IgE via IL-4, and IgG2b and IgG3 via TGF-β (Deenick, Hasbold *et al*., 2005). The extrafollicular reaction is rapid and short-lived as most cells die from apoptosis within a few days, leading to the production of Abs of germline affinity, in particular IgM, detectable at low levels in the serum. When some of these antigen-specific B cells reach specialized structures in the lymph nodes/spleen called germinal center (GCs) their differentiation into plasma cells producing high-affinity Abs takes place. In fact, B cells are attracted into GCs by antigen-specific T follicular helper (Tfh) cells that have upregulated C-X-C chemokine receptor type 5 (CXCR5), and by C-X-C motif chemokine 13 (CXCL13)-expressing follicular dendritic cells (FDCs). The interaction between B cells, Tfh and FDCs initiate the GC reaction, during which B cells receive additional activation and survival signals undergoing massive clonal proliferation. The intense proliferation is associated to two major events: Ig class-switch recombination from IgM towards IgG, IgA, or IgE, and maturation of the affinity for the specific antigen. This results into a massive production of Abs of a higher antigen binding ability. Since the development of the GC reaction generally requires a couple of weeks, hypermutated IgGs specific for protein antigens first appear in the blood 10-14 days after priming, with peak value usually at 4 weeks after immunization. Feedback mechanisms terminate GC reaction within maximum 6 weeks, a period during which a large number of antigen-specific plasma cells have been generated. At the end of this reaction, some plasma cells exit the lymph nodes/spleen and migrate to the bone marrow, where they survive through signals provided by supporting stromal cells. In secondary immune responses, booster exposure to antigen reactivates immune memory and results in a rapid increase of IgG titer (Plotkin, Orenstein *et al*., 2012) (**Fig. 4**).

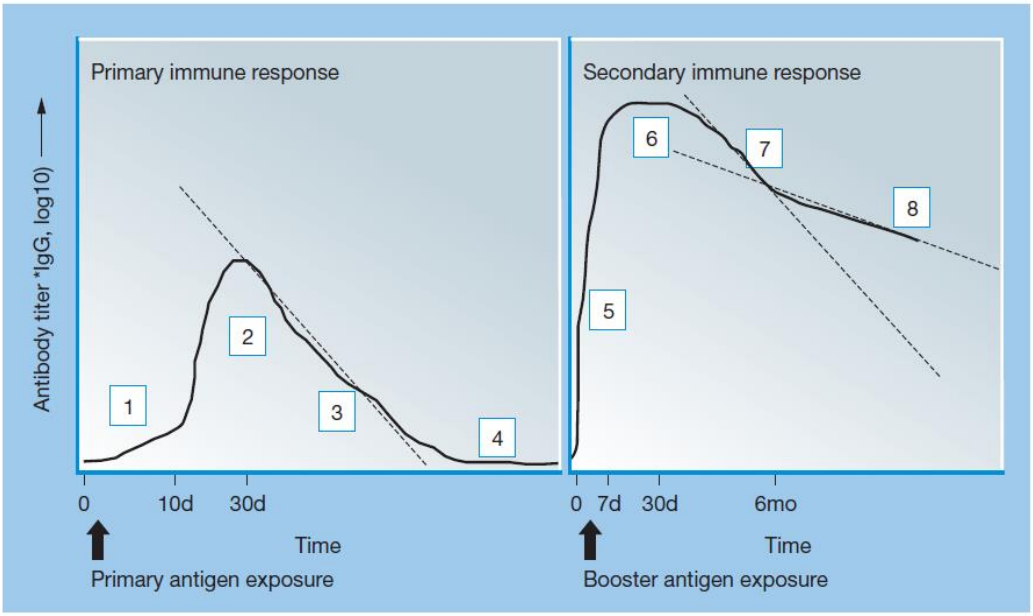


Figure 4. Correlation of IgG titers to the different phases of vaccine response. The first antigen exposure triggers an extrafollicular response (1) with production of Abs of germline affinity, with low levels of IgG. As B cells proliferate in GCs and differentiate into plasma cells, IgG titers reach a peak value (2); however, the short life span of these cells results in a rapid decline of the response (3), returning to baseline levels (4). In secondary immune responses, booster exposure to antigen reactivates immune memory B cells, resulting in a rapid increase of IgG titers (5). Short-lived plasma cells maintain peak of IgG levels (6) for a few weeks, which then decline initially with the same rapid kinetic observed with primary immunization (7), than slower as long-lived plasma cells that have reached survival niches in the bone marrow continue to produce antigen-specific Abs (8) (from Plotkin, Orenstein *et al*., 2012).

The intensity and duration of antibody responses is a function of numerous determinants:

a) the nature of the vaccine antigen and its intrinsic immunogenicity, which can be increased by vaccine adjuvants (Chowdhury and Ghosh, 2012);
b) the use of an optimal dose of vaccine antigen, which can be lower employing the right immunological adjuvant (Coffman, Sher *et al*., 2010);
c) genetic determinants (Plotkin, Orenstein *et al*., 2012);
d) number of vaccine doses, as very few protein-, polysaccharides-, or inactivated microorganisms-based vaccines induce high and sustained antibody responses after a single vaccine dose. The prime-boost strategy allows the presentation of high quantities of immunogen in the draining lymph nodes at several time points. The first dose initiates responses in which DCs and naïve immune cells are particularly involved, while boosts induce the activation of both effector and

memory cells (Wilson-Welder, Torres *et al*., 2009). Adjuvants permit immunization with fewer doses of vaccine (Coffman, Sher *et al*., 2010);

e) antigen persistence, which influences the generation of both memory T and B cells. Also in this case, antigen persistence can be improved by vaccine adjuvants (Awate, Babiuk *et al*., 2013).

3.1 Challenges and problems of cancer vaccines

The design of cancer vaccines is a great challenge for immunologists. In addition to the choice of the right antigen and adjuvant, the induction of the right type of immune response is crucial for tumor eradication. As the events regulating homeostasis of the immune system and the development of a protective immune response are coordinated to a large extent by cytokines produced by Th1 and Th2 lymphocytes, the role of Th1/Th2 balance has been widely investigated in cancer vaccine protocols, along with CTL and Abs activity.

Most clinical studies have demonstrated that alterations of Th1 and Th2 cytokine profile were usually characterized by decreased Th1/Th2 ratio in patients with different tumors including laryngo-pharyngeal HPV-positive (Bleotu, Chifiriuc *et al*., 2012), gastric (Ubukata, Motohashi *et al*., 2010), breast (Hong, Yao *et al*., 2013), bladder (Satyam, Singh *et al*., 2009), lung (Caras, Grigorescu *et al*., 2004), and pancreatic cancers (Tassi, Braga *et al*., 2009). Evidences obtained using a transgenic mouse model of breast cancer suggest that IL-4-mediated Th2 responses regulate the skewing of tumor-associated macrophages toward a type 2 (M2) phenotype (DeNardo, Barreto *et al*., 2009). In turn, M2 cells secrete immunosuppressive cytokines and promote tumor cell growth and invasion (Disis, 2010).

Data from 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 CTL response. For example, when compared to DNA vaccination, which does not generate detectable CTL activity, better antitumor immune responses have been obtained in BALB-neuT transgenic mice vaccinated with a HER2 peptide and a TLR agonist as adjuvant, since tumor protection and eradication appeared to be mainly due to the activation of a strong CTL activity (Nava-Parada, Forni *et al*., 2007). Moreover, Th1-specific immunity and in particular $CD8^+$ T cell recruitment have been demonstrated to correlate with better protective and therapeutic responses in patients with different tumor histotypes (Dredge, Marriott *et al*., 2002; Wang, Selleri *et al*., 2008).

Several data support the importance of the protective potential of Th1-isotype antibodies. In particular, in transgenic mouse models the IgG2a subclass appeared to play a pivotal role in tumor protection for anti-HER2 vaccination strategies, although it is still not clear whether the protective activity of IgG2a is dependent on complement-fixation activity, deprivation of HER2-mediated growth signals and/or other mechanisms. Moreover, some vaccination protocols in CTL-depleted BALB-neuT mice demonstrated that tumor protection appeared to be primarily mediated by antibody production (Nanni, Landuzzi *et al*., 2004; Park, Terabe *et al*., 2005). **Fig. 5** summarizes this Th1-skewed preferred scenario of immune responses triggered by a vaccine against a membrane oncoantigen, the most targeted antigen type in prophylactic cancer vaccinations.

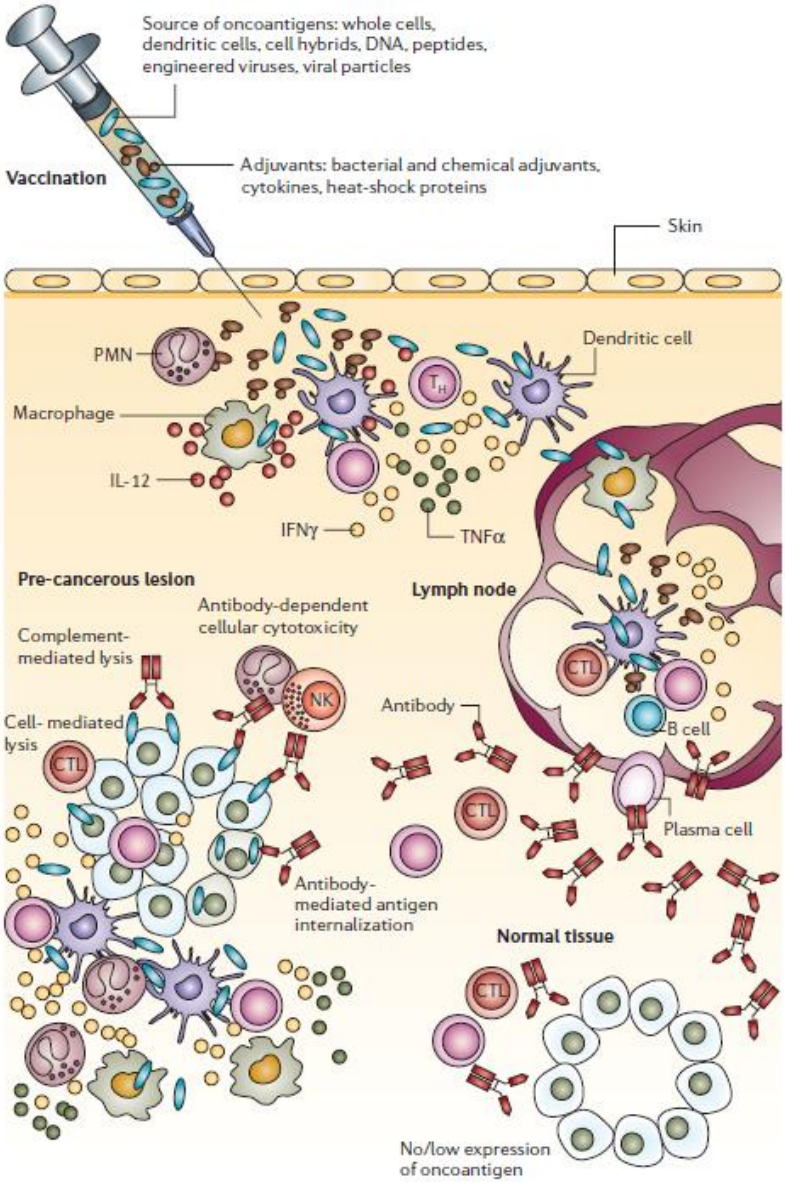


Figure 5. Vaccine-triggered Th1 immune responses against membrane oncoantigens. Administration of antigen and adjuvant triggers an inflammatory reaction that attracts polymorphonuclear cells (PMNs), macrophages, and DCs, which capture and process the antigen in order to present it to Th cells at the site of vaccine injection and/or in the draining lymph nodes. The activation of Th1 cells causes the release of IFN-γ that contributes to CTL activation and differentiation of B cells into plasma cells, which in turn produce different Abs and in particular IgG2a (in rodents). Th cells and CTLs migrate from the lymph nodes and infiltrate the precancerous lesion, recruiting activated PMNs and macrophages, and releasing TNFα and IFN-γ. These cytokines are cytostatic for tumor cells and increase MHC class I expression on both normal and tumor cells; moreover, IFN-γ blocks the tumor-driven angiogenesis and inhibits tumor invasion. Abs that recognize tumor antigen can trigger CDC, ADCC, antigen internalization and degradation, inhibit cell signaling, and/or block cell proliferation (from Lollini, Cavallo *et al*., 2006).

However, despite the importance of Th1-skewed responses in tumor vaccination, even Th2 cytokines and Abs can partly contribute to antitumor immunity (Ellyard, Simson *et al*., 2007). For example, although IL-10 is considered an inhibitor agent of TAA presentation by DCs, several preclinical models have shown that this Th2-type cytokine can also mediate tumor regression by stimulating NK cells and CTL activity (Mocellin, Marincola *et al*., 2004). Possibly, successful antitumor prevention and rejection reside in a fine balance between Th1 and Th2 responses, as recently demonstrated in a comparative study of the two HPV-vaccines Gardasil and Cervarix. This study has shown that even if both formulations efficiently induce protective immunity and diminish the associated cervical intraepithelial neoplasia in young women, Cervarix produces significantly higher titers (about 3.7-fold) of neutralizing Abs to HPV type 16 than does Gardasil, as well as more memory cells. Indeed, Gardasil contains only alum, with consequent Th2-skewed immune responses, while Cervarix contains AS04, an immune potentiator composed of alum and 3-O-desacyl-42-monophosphoryl lipid A (MPL), a TLR4 ligand that instead induces Th1 immunity (Giannini, Hannon *et al*., 2006). Hence, the design of more efficient and safer cancer vaccines requires further studies to better understand how Th1 and Th2 immunity are involved in immune-mediated tumor rejection.

Potential side effects represent another problem concerning cancer vaccines (**Fig. 6**).

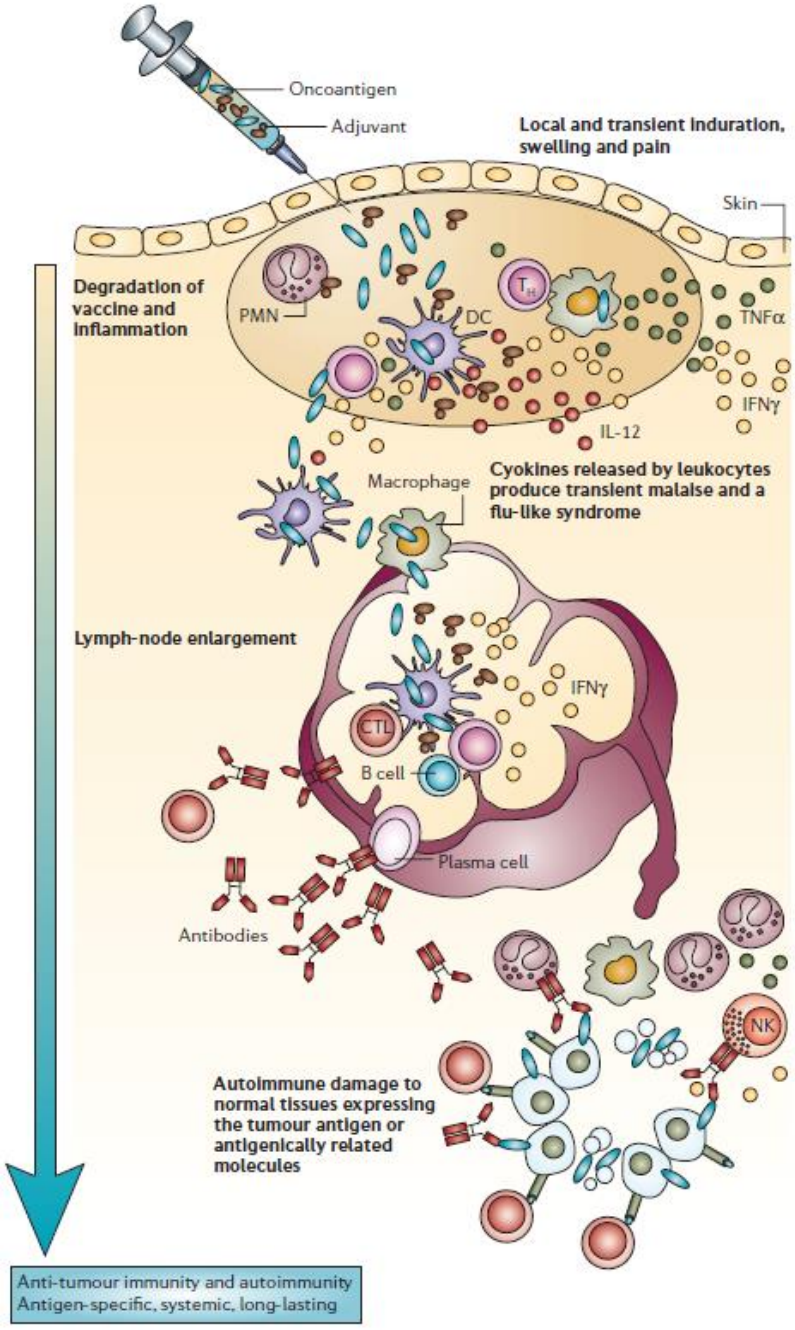


Figure 6. Side effects of cancer vaccination. Vaccination against tumor antigens can elicit undesired immune responses in the host. Degradation of vaccine components is accompanied by local inflammation whose degree is predominantly dependent on the type of immune adjuvant employed. Indeed, adjuvants determine the magnitude and type of both local reactions, which are mediated by infiltrating leukocytes, as well as systemic inflammation, which instead is provoked by cytokines that have reached the bloodstream. Mild adenopathy can develop in the draining lymph nodes as a consequence of antigen presentation, cell migration, and proliferation. Autoimmunity is the most severe side effect associated with vaccines

targeting antigens that are also expressed by normal tissues. Autoimmune reactions can be directed also against normal components antigenically related to the antigen but not included in the vaccine, a phenomenon called antigen spreading (from Lollini, Cavallo *et al*., 2006).

Although, in general, vaccines are among the safest products of modern medicine, they are constantly the subject of suspicious and apprehension (Ritvo, Wilson *et al*., 2005). In a patient with a life-threatening cancer, side effects of treatments are in general balanced by the antitumor benefits achieved; however, these effects are not acceptable for preventive vaccines, which are given to healthy people for long periods of their life. Inflammatory reactions that follow vaccine injection are usually local and transient. The release of proinflammatory cytokines such as IFN-γ or TNFα can sometimes induce a mild, systemic flu-like syndrome, characterized by fever, headache, fatigue, and musculoskeletal pain. The major cause of these side effects are adjuvants, in particular those employed for cancer vaccine protocols, which are much more potent, and thus much more toxic than those utilized for prevention of infectious diseases (Petrovsky and Aguilar, 2004; Lollini, Cavallo *et al*., 2006). However, repeated administrations of vaccine taper off this kind of side effects. The major problem concerning cancer vaccines is the fact that they target self-antigens and consequently they may induce autoimmune responses, which are dependent on both the type of targeted tumor antigen and of immune response elicited. Nevertheless, overexpression of oncoantigens by cancer cells and elicitation of low-avidity reactions in tolerant hosts render the immune reaction selective and reduce risks. For these reasons, preventive cancer vaccination strategies generally target oncoantigens, such as HER2. In fact, different preclinical and clinical studies confirm that no signs of autoimmunity were found after HER2-vaccination, supporting the evidences that low-avidity responses might be crucial in discriminating differences in HER2 expression between normal and tumor cells (Lo Iacono, Cavallo *et al*., 2005; Schiffman and Disis, 2010; Norell, Poschke *et al*., 2010).

Finally, as already mentioned, the additional challenge for therapeutic cancer vaccination is represented by ageing- and tumor-induced immunosuppression and immune evasion mechanisms. With few exceptions, patients with diagnosed cancer are of advanced age. Unfortunately, it has been demonstrated that a progressive immune deterioration occurs during ageing, and consequently the capacity to generate primary and memory immune responses is extremely compromised (Lu and Cerny, 2002; Fulop, Le Page *et al*., 2014). The immune impairment is mainly due to the age-dependent thymic involution that determines a defect of both T-cell mediated and T-cell dependent functions. Some strategies have been designed to overcome age-related problems, but with few results. For example, engagement of the

costimulatory molecule CD137 has shown to amplify T-cell responses in aged mice (Bansal-Pakala and Croft, 2002); moreover, some adjuvants may better stimulate immune responses in older, such in the case of the TLR ligand CpG, which seems to enhance Th1-cellular and humoral immunity in old mice (Maletto, Rópolo *et al*., 2005).

Immune suppression and immune evasion mechanisms are common problems that cancer vaccination has to deal with. The type and magnitude of the immune response generated by cancer vaccines is essential for avoiding such undesired mechanisms. As explained by the cancer immunoediting theory, the selective pressure exerted by the immune system, whether activated spontaneously or by vaccination, leads to the selection of tumor clones that are poorly immunogenic. Thus, an immune response that is not strong enough to eradicate a tumor, leads to the selection of tumor variants that are less sensitive to immune responses (Dunn, Bruce *et al*., 2002). In particular, when a therapeutic vaccination is administered all these mechanisms have already been established, and can be induced not only by tumor, but also by chemotherapy and age. The efficacy of preventive vaccines respect to the therapeutic ones is due to the fact that tumor cells have not accumulated enough genetic "hits" to evade immune system (Finn, 2014). In particular, vaccines targeting an oncoantigen that has not yet triggered tumorigenesis or is expressed only in a precancerous lesion can successful eradicate cells that proliferate at low rate, thus avoiding the possibility of clone selection and inhibiting lesion growth before complete neoplastic transformation takes place. Oncoantigens are suitable targets for tumor prevention as they permit to avoid tumor selection and escape, in particular because they regulate and control tumorigenesis. As the neoplastic process is dependent on these molecules, the probability of selection of tumor clones that have lost their expression is markedly reduced. Moreover, oncoantigens are expressed on the cell surface and are not impaired by down-modulation of MHC class I glycoproteins, a common mechanism by which tumor escape immune surveillance (Lollini, Cavallo *et al*., 2006).

4. Vaccine adjuvants

Very few antigens are intrinsically immunogenic and thus the majority of vaccine formulations requires adjuvants. Indeed, without a component able to engage both innate and adaptive immune cells, most non-adjuvanted and highly purified antigens induce tolerance rather than immunity (Matzinger, 1994). The word adjuvant comes from the Latin term "*adjuvare*", which means "to help" or "to enhance"; in fact, vaccine adjuvants are molecules, compounds or macromolecular complexes that enhance the magnitude, breadth, quality, and longevity of

specific immune responses to antigens, causing minimal toxicity or long lasting immune effects on their own. The addition of adjuvants to vaccines permits to reduce the amount of antigen and/or number of immunizations required to achieve and modulate the desired immune responses, improving also the efficacy in poor responder populations including newborns, immunocompromised individuals, and elderly (Petrovsky and Aguilar, 2004; Dubensky and Reed, 2010). Adjuvants have limited efficacy unless properly formulated and therefore both adjuvant components and formulation are crucial for enhancing vaccine potency (Reed, Bertholet *et al*., 2009).

The first observation of immune potentiation by adjuvants was made in 1893, when Coley observed that administration of killed bacteria (Coley's toxins) could in some cases cure certain forms of cancer. Only in the 1990s it was determined that this effect was due to immune stimulation mediated via bacterial DNA (Plotkin, Orenstein *et al*., 2012). It took another two decades to recognize the potential of adjuvants in enhancing humoral immunity, when in 1925 the French veterinarian Gaston Ramon noticed that the administration of diphtheria toxoid to horses 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 antigen alone (Ramon, 1925). A year later, Alexander Glenny and coworkers observed a similar effect with aluminum potassium sulfate, or alum (Glenny, Pope *et al*., 1926). Alum was used thereafter as an adjuvant in a wide number of human vaccines, and today in the form of aluminum oxyhydroxide or hydroxyphosphase is the only adjuvant approved for human use in the USA (Tritto, Mosca *et al*., 2009). Surprisingly, despite the wide use of alum and other adjuvants in human and animal formulations, their mechanisms of action are not fully characterized and this fact makes the adjuvants "the immunologists' dirty little secret" (Janeway, 1989). However, recent advances in immunobiological research have revealed several mechanisms by which adjuvants act, which are represented in **Fig. 7**.

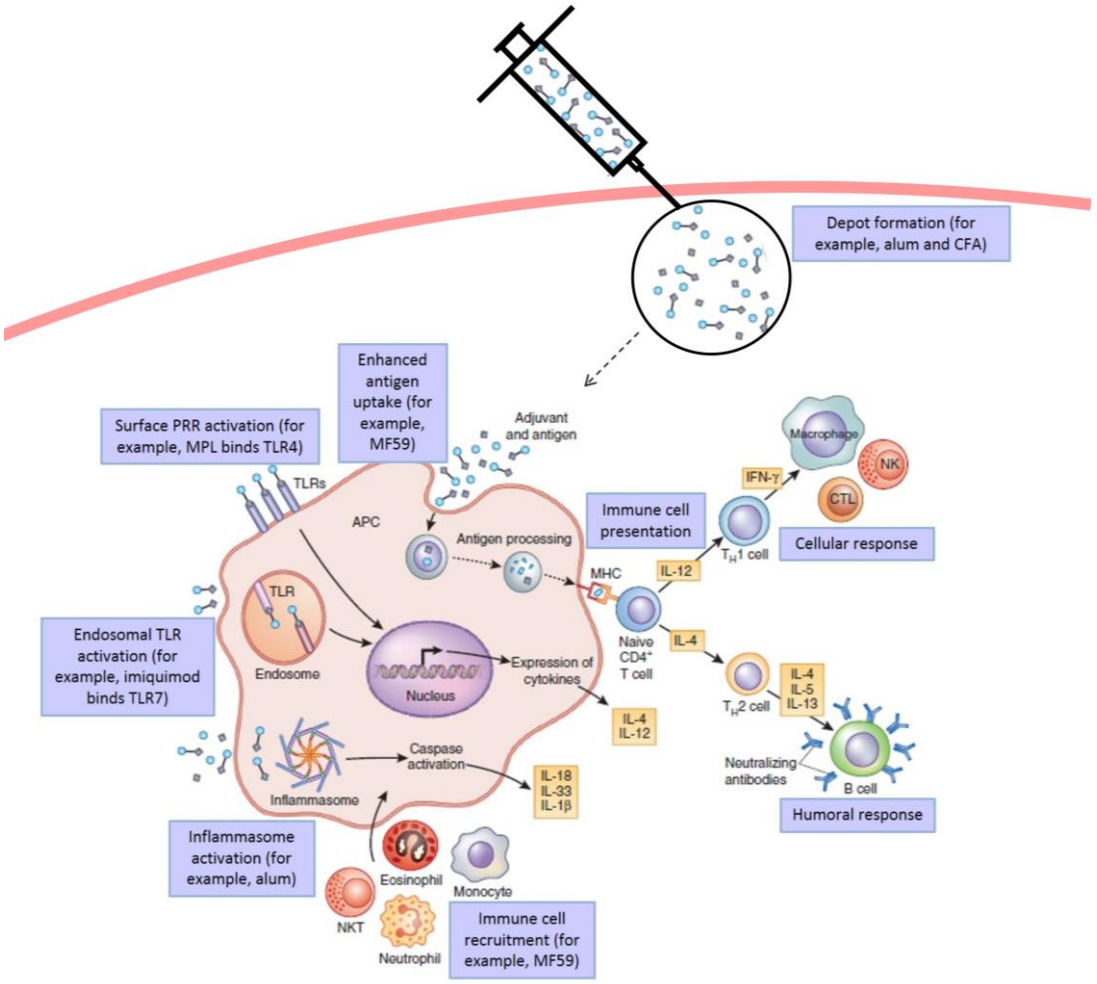


Figure 7. Proposed mechanisms of action of vaccine adjuvants. Adjuvants can mediate their activity through different mechanisms of action. Many adjuvants form a depot at the site of injection, which is associated with a slow release of antigen, and a persistent and more efficient presentation by APCs. Adjuvants can also create a proinflammatory microenvironment at the injection site with transient production of cytokines and chemokines, leading to recruitment of cells of the innate immune compartment. These cells express various PRR both on the surface and intracellularly, and after ligation of adjuvants to these receptors, they become mature and activated. Among recruited cells, DCs play a pivotal role in enhancing and influencing antigen-specific immune responses. In fact, DCs 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. Inflammasome activation has also been implicated as a mechanism of action for some adjuvants, leading to the production of proinflammatory cytokines IL-1β and IL-18 (adapted from Reed, Orr *et al*., 2013).

a) Sustained release of antigen at the site of injection (depot effect). The formation of a depot at the site of injection is the first and most widely recognized mechanism of action of vaccine adjuvants.

Antigen trapping and slow release at the inoculation site ensure a slow clearance of the antigen from the body, and a constant and more efficient stimulation of the immune system (Awate, Babiuk *et al*., 2013; Wilson-Welder, Torres *et al*., 2009). This can be achieved as a short-term or long-term depot, the latter giving either a continuous or pulsed release. Short-term depots are formed by aluminum salts and water-in-oil (w/o) emulsions, such as incomplete Freund's adjuvant (IFA), where antigen is trapped at the injection site and cannot be lost by liver clearance. Long-term depots are achieved through synthetic polymers such as polylactide coglycolide (PLG) injection, producing microspheres that degrade to yield a pulsed delivery (Cox and Coulter, 1997).

b) Cellular recruitment at the site of injection. Adjuvants create a local proinflammatory environment, with a consequent production of cytokines and chemokines that trigger the recruitment and activation of innate immune cells (Awate, Babiuk *et al*., 2013). For example, after intramuscular (i.m.) administration in mice, both alum and MF59 adjuvants strongly and rapidly recruit neutrophils, followed by monocytes, eosinophils, and DCs. Neutrophils are the most numerous cells to be recruited after vaccine injection; however, ablation of these cells appeared not to affect the immunogenicity of MF59 (Calabro, Tortoli *et a*l., 2011). In contrast, the depletion of $CD11c^+$ monocytes and DCs during immunization with an alum-based vaccine abrogated both antibody and cellular responses, suggesting that these cells play a central role in immune response induction (Lambrecht, Kool *et al*., 2009). Similar to MF59, administration of AS03, a squalene-based oil-in-water (o/w) adjuvant, leads to enhanced recruitment at the site of injection of neutrophils, monocytes, and eosinophils, which take up antigens and traffic towards the draining lymph nodes (Morel, Didierlaurent *et al*., 2011).

c) Immunomodulation. This word refers to the ability of many adjuvants to modify the cytokine network, influencing the balance between Th1 and Th2 responses, and the polarization of T cell responses towards cellular or humoral responses. In fact, immunomodulation may result in a general activation of the entire immune system; however, depending on the type of adjuvant, it induces upregulation of certain cytokines and chemokines and a concomitant downregulation of others. Therefore, selection of the appropriate immunomodulatory adjuvant will not only lead to enhanced immune response but will also determine the isotype of IgG and the magnitude of cell-mediated immunity generated (Cox and Coulter, 1997; Reed, Orr *et al*., 2013). The most notable example of Th-skewed response is induce by alum, which in humans promotes more than 90% of Th2-type immune responses, resulting in robust antibody production (Cox and Coulter, 1997; Reed, Bertholet *et al*., 2009). The majority of current licensed vaccines are not able to induce Th1-

type immune responses without generating at the same time undesirable toxic side effects (Wilson-Welder, Torres *et al*., 2009).

d) Increased antigen uptake and presentation by APC. Induction of a potent immune response requires an efficient antigen presentation by APCs (Awate, Babiuk *et al*., 2013). Many adjuvants such as alum and oil-based emulsions act targeting antigens to APCs, resulting in enhanced antigen presentation by MHC (Awate, Babiuk *et al*., 2013). In fact, it has been shown that antigen adsorption on alum results in an increased internalization of the antigen itself (Morefield, Sokolovska *et al*., 2005); however, alum does not enter DCs directly, but probably delivers the antigen via abortive phagocytosis, after interaction with membrane lipids on DCs and recruitment of immunoreceptor tyrosine-based activation motif (ITAM) containing molecules (Flach, Ng *et al*., 2011).

e) Activation and maturation of APCs. Activation of APCs, in particular DCs, is essential for induction of adaptive immune responses, and results in an increased expression of MHC class II, activation marker CD68, and maturation marker CD83, which lead to enhanced ability to induce T lymphocyte activation and differentiation (Coyle and Gutierrez-Ramos, 2001). As already described, antigen recognition through PRRs, and in particular TLRs, triggers DCs maturation (Pardoll 2002), and consequently most studies are focused on improving vaccine formulations by the addition of TLR ligands as adjuvants. For example, AS04 has been shown to interact with TLR4, inducing maturation of DCs, which then traffic to the draining lymph nodes to activate antigen-specific T cells (Didierlaurent, Morel *et al*., 2009). Interestingly, *in vitro* studies on human cells have shown that both alum and MF59 failed to directly activate DCs, but enhanced the surface expression of MHC class II and costimulatory molecules on monocytes, macrophages, and granulocytes, thus resulting in an increased proliferation of responding T cells (Seubert, Monaci *et al*., 2008). However, using a transgenic mouse model for diphtheria toxin-mediated DC ablation, it has been found that DCs are indispensable for alum adjuvanticity (Kool, Soullié *et al*., 2008). These observations suggested that both adjuvants may act upstream of dendritic cells and impact instead on monocytes recruitment and monocytes-to-DC differentiation (Seubert, Monaci *et al*., 2008; Calabro, Tortoli *et a*l., 2011).

f) Activation of inflammasomes. 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). ASC protein can recruit caspase 1, thereby linking the PRR to caspase 1 activation and production of IL-1β and IL-18 that are potent stimulators of the adaptive immunity.

There are currently four characterized inflammasomes, named by the PRRs that form them: NLPR1 (NOD-, leucine-rich repeat (LRR)- and pyrin domain-containing 1), NLRP3, NLRC4 (NOD-, LRR- and CARD-containing 4), and AIM2 inflammasomes. Among them, NLRP3 and AIM2 sense also DAMPs (Chen and Nuñez, 2010). In particular, it was proposed 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. In fact, secretion of uric acid forms monosodium ureate crystals whose phagocytosis result in phagosomal destabilization and lysosomal rupture, releasing the protease cathepsin B in the cytosol, which in turns activate NLRP3 (Hormung, Bauernfeind *et al*., 2008). Similarly, ATP released by damaged cell has been shown to directly activate NLRP3 (Pétrilli, Papin *et al*., 2007). Although *in vitro* data supporting NLRP3 inflammasome activation by alum are convincing, there is controversy about the role of this pathway *in vivo* (Bergmann-Leitner and Leitner, 2014).

Most of licensed adjuvants were developed using empirical methods, thus they are not optimal for many of the challenges of vaccination, especially in the oncologic field. In particular, the historical emphasis on humoral responses has favored the development of adjuvants with the ability to mainly enhance antibody production. Consequently, most of the licensed adjuvants are effective in enhancing antibody titers, but do not elicit strong Th1 and/or CTL responses (Pashine, Valiante *et al*., 2005; Levitz and Golenbock, 2012). To solve this problem, new generation vaccines often incorporate agonists of TLRs and/or other innate immune receptors to facilitate the generation of cellular responses (Reed, Orr *et al*., 2013). **Table 3** summarizes the main characteristics of adjuvants licensed for human use or that are in advanced stage of clinical development.

Adjuvant name (year licensed)	Formulation	Mechanism of action	Type of immune response	Licensed product name/type of vaccine in clinical trials
Licensed human adjuvants				
Alum (1924)	Mineral salts: aluminum phosphate or hydroxide	Nalp3, ITAM, antigen depot, antigen delivery, cellular recruitment	Abs, Th1 (+/-) Th2 (+++)	Various, *e.g.*, HBV, HPV, tetanus, diphteria

MF59 (Novartis, 1997)	o/w emulsion: squalene, polysorbate 80 (Tween 80), sorbitan trioleate 85 (Span 85)	No antigen depot effect, cell recruitment, antigen uptake	Abs, Th1 (+), Th2 (++)	Fluad (seasonal influenza), Focetria (pandemic influenza), Aflunov (pre-pandemic influenza)
AS03 (GlaxoSmithKline, 2009)	o/w emulsion: squalene, Tween 80, α-tocopherol	Cell recruitment, antigen presentation, antigen uptake	Abs, Th1 (+), Th2 (++)	Pandremix (pandemic influenza), Prepandrix (pre-pandemic influenza)
Virosomes (Berna Biotech, 2000)	Liposomes: lipids, hemagglutinin	PAMP signals, binding to APCs and inducement of receptor-mediated endocytosis, antigen presentation via MHC class I and II	Abs, balanced Th1 and Th2	Inflexal (seasonal influenza), Epaxal (hepatitis A)
AS04 (GlaxoSmithKline, 2005)	Alum-adsorbed TLR4 agonist: aluminum hydroxide, MPL	TLR4 agonist, activation of NF-κB pathway, cell recruitment	Abs, Th1 (++), Th2 (+)	Fendrix (HBV), Cervarix (HPV)
Adjuvants in advanced stage of clinical development				
CpG-oligodeoxynucleotides (ODN) (CpG 7909; CpG 1018)	CpG ODN alone or combined with alum/emulsions	TLR9 agonist	Abs, Th1, $CD8^+$ cells if conjugated	Seasonal influenza, HBV, melanoma

Polyiosinic:polycytidylic acid [Poly(I:C)]	Double-stranded RNA (dsRNA) analogues	TLR3 agonist	Abs, Th1, $CD8^+$ cells	Melanoma, prostate and triple-negative breast cancers
Imidazoquinolines (imiquimod, resiquimoid)	Small molecules	TLR7 and TLR8 agonists	Abs, Th1	Melanoma
AS01	Liposome, MPL, saponin (QS21)	QS21: unknown MPL: TLR4 agonist	Abs, Th1, $CD8^+$ cells	Malaria, HIV, breast, prostate, lung cancers
AS02	o/w emulsion; MPL, saponin (QS21)	QS21: unknown MPL: TLR4 agonist	Abs, Th1	Malaria, tuberculosis, non-small-cell lung cancer, melanoma
Immunostimulatory complexes (ISCOMs) and ISCOMATRIX	Saponin, cholesterol, dipalmitoylphosphatidylcholine	Unknown, probably independent from PRRs	Abs, balanced Th1 and Th2, $CD8^+$ cells	Pandemic influenza, HIV, HBV, malaria, melanoma

Table 3. Formulation, mechanisms of action, and applications of adjuvants that are worldwide currently licensed for use in humans or that are in clinical development (adapted from Reed, Bertholet *et al*., 2009; Coffman, Sher *et al*., 2010; Nicholls, Madera *et al*., 2010; Rappuoli, Mandl *et al*., 2011; Awate, Babiuk *et al*., 2013; Reed, Orr *et al*., 2013).

4.1 Aluminum adjuvants

Aluminum salts-based adjuvants, generally referred to as alum, are non-crystalline gels based on aluminium oxyhydroxide (aluminium hydroxide gel), aluminium hydroxyphosphate (aluminium phosphate gel), or various proprietary salts such as aluminium hydroxyl-sulfate. These adjuvants are components of several licensed vaccines, including vaccines 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 HPV (Reed,

Bertholet *et al*., 2009; Mbow, De Gregorio *et al*., 2010). The alum-based vaccine formulation is prepared by suspending the antigen in a phosphate buffered solution and then allowing it to adsorb to the aluminium hydrogel. The amount of antigen that adsorbs onto alum depends upon the forces within the antigen, and between the antigen and the alum, including hydrophobic interactions, van der Waals forces, ionic charges, and hydrogen bonding (Wilson-Welder, Torres *et al*., 2009).

Despite alum has long been used in vaccines and thus its activity and safety profile is extensively documented, its multiple potential mechanisms of action are only now beginning to be elucidated, with sometimes conflicting evidences. In 2008, De Gregorio *et al.* firmly supported the idea that alum enhances antigen uptake by DCs, cell recruitment at the injection site, and stimulation of immune cells via inflammasome. However, there were disputes on the specifics of NLRP3 inflammasome activation and its central role in alum adjuvanticity, with additional discrepancies between *in vitro* and *in vivo* responses (De Gregorio, Tritto *et al*., 2008), as already described above. In 2011 Marichal *et al*. proposed that alum adjuvanticity is specifically related to the release of DNA from necrotic cells exposed to alum (Marichal, Ohata *et al*., 2011). Despite these divergences, it has been widely accepted that one of the possible reasons for alum Th2-biased responses might be the NALP3-dependent induction of IL-1β, IL-18, and possibly IL-33 (Lambrecht, Kool *et al*., 2009). Other mechanisms of action of alum have been proposed during the last years, including the already mentioned observations of Flach *et al*. (Flach, Ng *et al*., 2011). Moreover, the study of Shah *et al*. showed that type II NKT cells are involved in alum adjuvant activity in a CD1d-dependent manner and mediated by Th2 cytokine production (Shah, Devera *et al*., 2012), while the work of Wang *et al*. confirmed the integral role of inflammasomes, but suggested that this response is mediated by heat shock protein 70, thus indicating that alum acts as a stress-inducing agent (Wang, Rahman *et al*., 2012). The interpretation of all these reports is complicated by the lack of uniformity in the available reagents classified as aluminum salts, the animal models used, the antigen dose, and the immunization regimens (Reed, Orr *et al*., 2013; Bergmann-Leitner and Leitner, 2014). However, taken together, some common themes do emerge: alum affects antigen uptake, induces danger signals, recruits various types of immune cells, and elicit powerful Th2-biased humoral responses (Reed, Orr *et al*., 2013).

The major limitations of aluminum adjuvants include their inability to elicit cell-mediated Th1 or CTL responses that are required to control most intracellular pathogens such as those that cause tuberculosis, malaria, leshmaniasis, AIDS, and other pathologies such as cancer (Reed, Bertholet *et al*., 2009). Indeed, alum is not ideal for small peptide vaccines or for use with recombinant

proteins due to their inherent low immunogenicity. In addition, alum cannot be employed for oral or intranasal immunization, and vaccines containing this adjuvant cannot be lyophilized or frozen because this leads to loss of potency, thus limiting vaccines shelf life and storage conditions. Finally, alum has been associated with severe tissue reactions such as erythema, subcutaneous nodules, granulomas, and has been thought to induce hypersensitivity and macrophagic myofasciitis. Moreover, alum-based vaccines can induce IgE and IL-4, which are associated with allergy and type I immediate hypersensitivity (Wilson-Welder, Torres *et al*., 2009; Adams and Mallapragada, 2014). However, IgE-mediated allergic reactions to aluminum-adjuvanted antigens have been rarely reported in vaccinated individuals and, by contrast, aluminum hydroxide has been used in allergen immunotherapy to reduce allergen-specific IgE responses (Aimanianda, Haensler *et al*., 2009).

4.2 Squalene-based adjuvants: MF59 and AS03

MF59 consists of an o/w nano-emulsion composed of <250 nm droplets. MF59 contains squalene, which derives from biodegradable plant oil, and Tween 80 and Span 85 as stabilizers (Wilson-Welder, Torres *et al*., 2009). It has been used in Europe as an adjuvant in influenza vaccines for its capacity to increase flu immunogenicity in the elderly and young children (Mbow, De Gregorio *et al*., 2010). Overall, MF59 has an acceptable safety profile, and generates higher antibody titers with more balanced IgG1:IgG2a responses than those obtained with alum. However, as for alum, MF59 stimulates strong Th2-biased immune responses, and therefore it may not be suitable for vaccines requiring cell-mediated immunity for protection. The mechanisms of MF59 adjuvanticity, similarly to alum, seems to be in part due to direct delivery of immunogens to APCs *in vivo*; however, it has been demonstrated that antigen biodistribution and clearance from the site of injection are not influenced by MF59, suggesting that the adjuvant does not induce any antigen depot (Dupuis, McDonald *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 antigen to the draining lymph nodes, thus resulting in a more efficient T cell priming (Dupuis, Denis-Mize *et al*., 2001). Additionally, MF59 triggers a local immunostimulatory microenvironment characterized by the expression of several cytokines, which may enhance and accelerate the differentiation of monocytes towards DCs (Tritto, Mosca *et al.*, 2009). Interestingly, the adjuvanticity of MF59 seems to be due to the o/w formulation, and not to the individual components, suggesting that the formulation itself plays a pivotal role in eliciting strong immune responses. Indeed, all components are necessary to create the stable

small emulsion droplets that are necessary for an effective MF59 adjuvant, since the elimination of any individual component induces an evident decrease in the adjuvant effect (Calabro, Tritto *et al*., 2013).

AS03, another squalene-based o/w emulsion, differs from MF59 as it contains α-tocopherol, whose presence modulates the cytokine response at the injection site and cell recruitment to the draining lymph nodes, resulting in an enhanced antibody response. Comparing o/w emulsion with alum, MF59 and AS03 turned out to be superior in terms of induction of protective Abs, increased sero-conversion, and cross-protection (Schwarz, Horacek et al., 2009). MF59- and AS03-adjuvanted influenza vaccines have been approved for use in Europe and Canada, and both adjuvants allow antigen sparing, an important factor particularly during pandemics (Levitz and Golenbock, 2012).

4.3 Virosomes

Virosomes are particles composed of stabilized membrane lipids and functional viral fusion proteins that can be used to deliver vaccine antigens (Wilson-Welder, Torres *et al*., 2009). The majority of virosomes comprises influenza hemagglutinin (HA) and neuraminidase proteins, and can be generated by inserting the viral fusion proteins and antigen into small phospholipid vesicles or by separation and reconstitution of viral envelopes with the vaccine antigen. Virosomes maintain the receptor binding ability and mimic infectivity of the native virus, but without the risks associated with the virus itself; moreover, they can directly deliver the antigen into the cytosol of target cells, in particular through HA receptor-mediated endocytosis by APCs. Indeed, this technique permits the induction of both humoral and cellular responses because some of the virosome-delivered antigens have the potential to be presented via MHC II following endosomal processing; alternatively, virosomes that enter the cytosol will be presented via MHC I (Wilson-Welder, Torres *et al*., 2009; Reed, Bertholet *et al*., 2009).

4.4 Saponin-based adjuvants, ISCOMs and ISCOMATRIX

Saponins are natural detergent-like molecules that can induce both humoral and cellular immunity, but can also be haemolytic and cytotoxic towards human cells (Nicholls, Madera *et al*., 2010). Quil A is composed of different saponins from the bark of the South American tree *Quillaja saponaria*, and has shown good promise in veterinary vaccination protocols; however, it is still too toxic for use in humans (Sun, Xie *et al*., 2009). The saponin derivative QS21 is far less toxic than Quil A, and is a good inducer of Th1 responses; moreover, it seems to improve antigen

presentation and promote cell-mediated immunity. As a result of promising preclinical data, GlaxoSmithKline has developed two adjuvants formulations, AS01 and AS02, which both incorporate QS21 and are now evaluated in different clinical trials (Tagliabue and Rappuoli, 2008).

ISCOMs are cage-like nanoparticles composed of saponins purified from *Quillaja saponaria*, formulated with cholesterol, phospholipid, and antigen. The classical ISCOM technology required incorporation of vaccine antigens into the structure, which not only restricted the types of antigens that could be used, but was also a laborious process difficult to control. For these reasons, most current applications employ a mixture of soluble antigens and the antigen-free particle, such as ISCOMATRIX (Coffman, Sher *et al*., 2010). The mechanism of adjuvanticity of ISCOMs and ISCOMATRIX is still unclear; however, it seems to be independent from PRRs interaction. Probably, ISCOMs act by enhancing antigen uptake and prolonging retention of DCs in draining lymph nodes, inducing activation of DCs, thus leading to strong antibody and T cell responses (Maraskovsky, Schnurr *et al*., 2009). Of note, unlike most other adjuvants, ISCOMs and ISCOMATRIX enable substantial MHC class I and II presentation and induce both $CD4^+$ and $CD8^+$ T cell responses to different types of soluble protein antigens in humans (Davis, Chen *et al*., 2004; Morelli, Becher *et al*., 2012).

4.5 The ideal vaccine adjuvant

An ideal vaccine adjuvant must bridge the gap between innate and adaptive immunity, in order to promptly engage the immune system, and elicit a robust protective and/or therapeutic response also against future infections and disease relapses (Adams and Mallapragada, 2014). The ideal adjuvant must be safe, stable before administration under a broad spectrum of storage time/temperature/pH conditions, readily biodegraded and eliminated in order to decrease the risk of late adverse effects. Moreover, it must be also chemically and physically defined to ensure reproducible manufacturing and activity, and inexpensive to produce (Reed, Bertholet *et al*., 2009). Dose sparing is also required, in particular for antigens difficult to manufacture or that must be prepared on short notice, along with dosage sparing, which carries the great challenge of reaching complete immune protection with a single dose. Finally, the ideal vaccine adjuvant must overcome immune tolerance and senescence, two critical problems in the case of anticancer vaccines (Alving, Peachman *et al*., 2012).

None of the commercial adjuvants is able to fulfill all the characteristics of the ideal adjuvant (listed in **Fig. 8**), and the development of new vaccine adjuvants have been the focus of intense research efforts. In recent years, increasing evidence demonstrates that TLR ligands are safe and

effective compounds that can be exploited in the antitumor vaccination field (Smyth, Garcia *et al*., 2013).

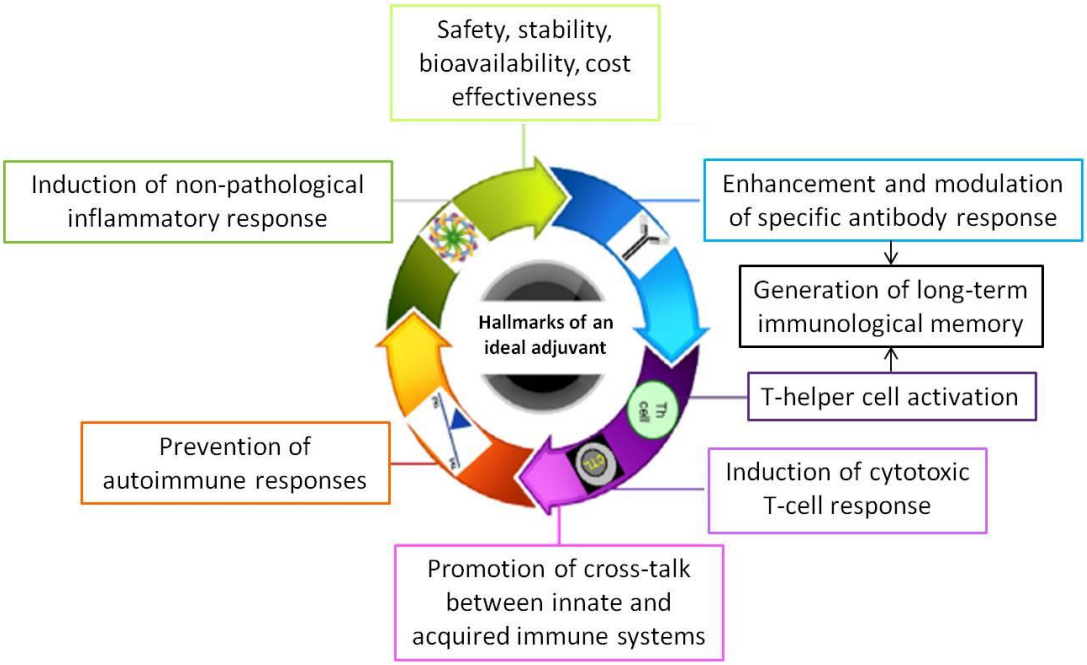


Figure 8. Schematic diagram showing the most important hallmarks of an ideal vaccine adjuvant (modified from Chowdhury and Ghosh, 2012).

4.6 TLR agonists: a new promising vaccine adjuvants group

TLR ligation was found to activate and modulate immune responses by increasing the crosstalk between innate and adaptive immunity, and to play an important role in antitumor immunity. Therefore, a new class of immunological adjuvants that targets the TLR pathways is emerging as an important element in the design of more effective and safer vaccine formulations (Ito and Chang, 2013).

Indeed, many successful vaccines contain motifs that are now known to stimulate TLR pathways (Mbow, De Gregorio *et al*., 2010). TLRs regulate immune responses through activation of different immune cell signaling. For example, the yellow fever vaccine, one of the most effective vaccines, activates multiple subsets of DCs by signaling through TLR2, 7, 8, and 9 (Querec, Bennouna *et al*., 2006). In B cells, TLR signaling induces upregulation of different surface markers involved in cross talk with T cells, namely MHC-I and -II, CD40, CD80, and CD86, thus ultimately enhancing antigen-specific antibody production. Moreover, TLR signaling is involved in induction

of B- and T-cell memory. In APCs, TLR activation enhances secretion of both pro- and anti-inflammatory mediators that drive development of Th cell subsets into Th1, Th2 or Treg responses, depending on the type of TLR involved (**Fig. 9**). Thus, TLR activation pathways can be exploited in the context of vaccination to favor antigen-specific Th-skewed immune responses (Smyth, Garcia *et al*., 2013; Toussi and Massari, 2014). Of note, TLR signaling can lead to either Treg functional activation or suppression, depending on the type of TLR and ligand involved, and thus Treg-mediated peripheral tolerance can be overcome by stimulation of TLRs, in particular those expressed by DCs (van Duin, Medzhitov *et al*., 2006). In fact, different studies have shown that TLR-mediated upregulation of costimulatory molecules play a pivotal role in the reversal of peripheral tolerance, thus leading to efficient CTL responses against tumor cells (Pasare and Medzhitov, 2003; Yang, Huang *et al*., 2004; Peng, Guo *et al*., 2005; Tacken, Zeelenberg *et al*., 2011).

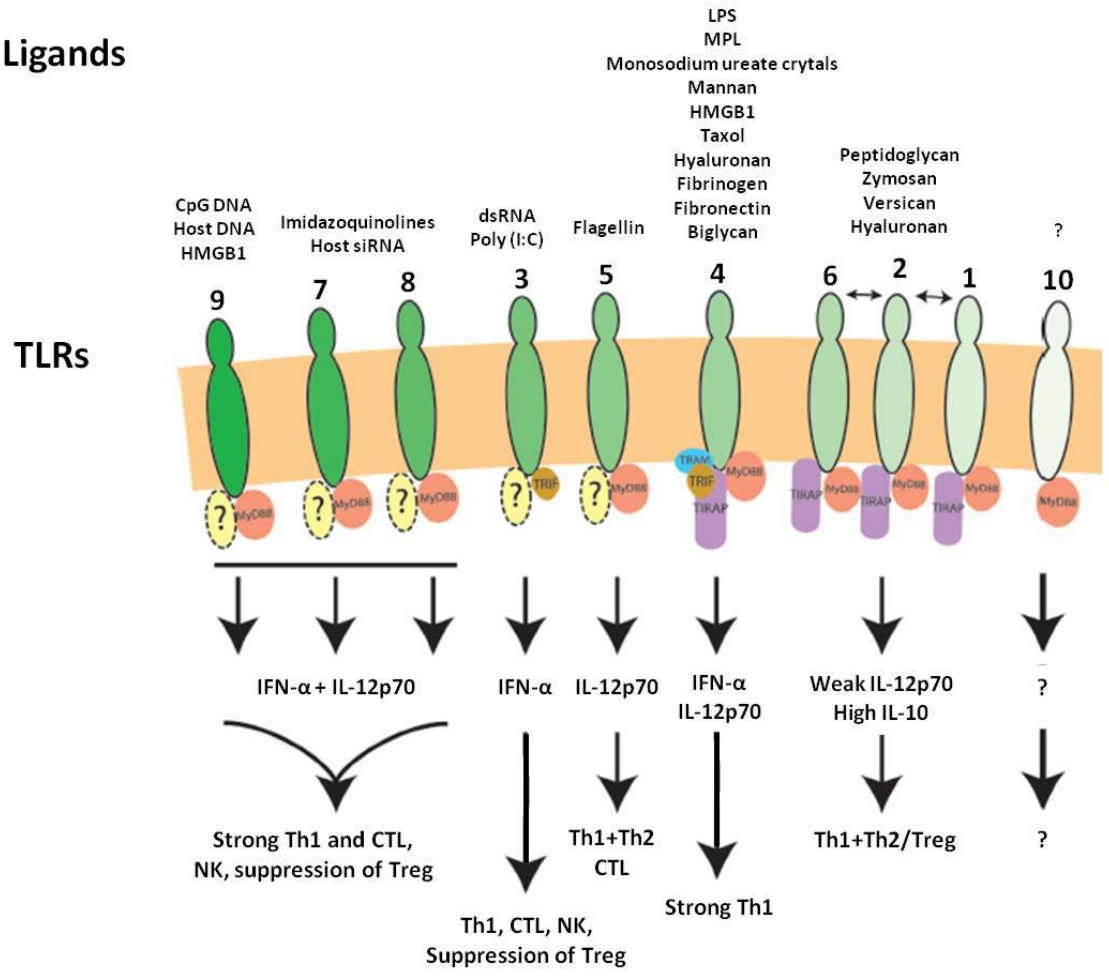


Figure 9. Regulation of adaptive immune responses by TLRs after interaction with their ligands. Triggering DCs through TLRs results in the induction of distinct adaptive immune responses. DCs triggering via TLRs 9,

7, 8, and 3 induces strong Th1 and CTL responses, while via TLR4 only Th1 responses. Activation of TLR5 enhances T cell and antibody responses without altering the Th1/Th2 balance. In some cases, TLR5 ligands induce also CTL responses. Conversely, DCs triggered via TLR2 heterodimers produce high levels of IL-10 and shift the balance towards the Th2/Treg spectrum; however, some ligands of TLR2 induce strong antibody responses without altering the Th1/Th2 balance (Coffman, Sher *et al*., 2010; Pulendran, 2004; Baxevanis, Voutsas *et al*., 2013. Adapted from Pulendran, 2004).

It has also been reported that the physical association of antigen and TLR agonist may be important for optimal antigen-specific immune responses, as DCs seem to preferentially process and present antigens from compartments that also contain TLR ligands (Blander and Medzhitov, 2006). Finally, combinations of TLR agonists can have synergistic effects, resulting in greater and more durable responses to antigens, and therefore in dose sparing (Levitz and Golenbock, 2012). A number of these agonists are now in clinical or advanced preclinical stages of development for application as vaccine adjuvants (Cheng, Jain *et al*., 2011; Offersen, Melchjorsen *et al*., 2012; Ghose, Verhagen *et al*., 2013; Tougan, Aoshi *et al*., 2013; Orr, Beebe *et al*., 2014), and have been extensively investigated to clarify the basis of their adjuvant activity (Coffman, Sher *et al*., 2010). Among TLR agonists, NPs and their derivatives from plants, animals, and microbes that can act as PAMPs or DAMPs, have been studied for many years as adjuvants since they offer a unique set of advantages over conventional adjuvants. In fact, NPs have been already widely employed in different clinical settings for long-acting delivery of nucleotide, peptide, and protein therapeutics, showing to be cheap, biocompatible, biodegradable, non-toxic, non-immunogenic and non-inflammatory (Adams and Mallapragada, 2014). As vaccine adjuvants, NPs have showed the ability to efficiently activate DCs, resulting in long lasting humoral and cellular immune responses, thus being promising for the development of single dose vaccines due to their ability to sustain the release of the antigen over an extended period of time. Among NPs, zymosan, chitosan, mannan, and poly-γ-glutamic acid are showing good promise as vaccine adjuvants, and MPL has been approved for human use in different types of vaccine formulations, such as the anti-HBV vaccine Fendrix, and the anti-HPV vaccine Cervarix (Shakya and Nandakumar, 2012; Wilson-Welder, Torres *et al*., 2009).

4.6.1 TLR4 agonists: from LPS to MPL and AS04

It is widely known that lipopolysaccharide (LPS) is able to directly induce DC maturation and also strongly stimulates a variety of cells to produce cytokines and chemokines that control DC trafficking and maturation (Banchereau and Steinman, 1998). In particular, LPS stimulates robust

IL-12 production in DCs *in vitro*, and thus it would be predicted to promote Th1-type responses *in vivo* (Brightbill, Libraty *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. Most of the immunomodulatory effects of LPS derives from its lipid A portion, which is located in the outer membrane of gram-negative bacteria (Persing, Coler *et al*., 2002). To solve this issue, a non-toxic derivative of the lipid A portion of LPS of *Salmonella minnesota*, namely MPL, was obtained by removing a phosphate group, the sugar moiety, and an ester-linked fatty acid group. MPL has been shown to possess many adjuvant properties of LPS, as it interacts with TLR4 on APCs, triggers the synthesis of IL-1β, IL-12 and IFN-γ, and induces DCs maturation, migration, and efficient priming of naïve T cells (Wilson-Welder, Torres *et al*., 2009; Tritto, Mosca *et al*., 2009). In animal studies, MPL demonstrated to be a potent stimulator of Th1 and CTL responses. Moreover, MPL enhances the production of complement-fixing Abs and secretory IgA immunoglobulins (McNeela and Mills, 2001).

MPL adsorbed to alum, known as AS04, is approved for use in vaccines against HBV and HPV. It induces a transient and local activation of NF-κB activity and cytokine production, thus providing an innate immune signal for optimal activation of APCs (Mbow, De Gregorio *et al*., 2010). Interestingly, AS04 stimulates a polarized Th1 cell response in contrast to the strong Th2 response induced by alum alone (Casella and Mitchell, 2008; Didierlaurent, Morel *et al*., 2009). No synergistic effect between MPL and alum was in general observed; however, Didierlaurent *et al.* suggested that alum might prolong the cytokine response induced by MPL at the injection site (Didierlaurent, Morel *et al*., 2009).

MPL is also a component of two experimental adjuvants, AS01 and AS02: the first contains liposomes, MPL and QS21, whereas AS02 is an o/w emulsion containing MPL and QS21. AS02 shows promise in cancer vaccination strategies, since it elicits both humoral and cell–mediated immune responses, by mediating both high antibody titers and IFN-γ production. AS01 promotes a stronger Th1 response, by inducing a transient stimulation of the innate immune system that leads to the generation of high number of efficient antigen-presenting DCs, as it has been demonstrated in clinical trials (Garçon, Chomez *et al*., 2007).

4.6.2 TLR9 agonists: CpG ODN

TLR9 agonists represent one of the most advanced candidates as vaccine adjuvants, since TLR9 engagement efficiently induces INF-γ production by T cells, thereby resulting in strong antibody and Th1-biased T cell responses (Dougan and Dranoff, 2009; Mbow, De Gregorio *et al*., 2010;

Levitz and Golenbock, 2012). In humans, TLR9 is expressed on plasmacytoid dendritic cells (pDCs) and B cells. Moreover, TLR9 is the only endosomal PRR specific for DNA and mediates a potent innate response to bacterial and viral DNA (Blasius and Beutler, 2010). A variety of synthetic TLR9 agonists have been developed, and are under investigation against a wide range of tumors (Dougan and Dranoff, 2009). In particular, synthetic immunostimulatory sequences (ISS) containing 15-20 base ODN with optimized repeated sequences of CpG, named CpG-ODN, have been studied extensively as adjuvants, either soluble or embedded in nanoparticles or virus-like particles (Marshall, Higgins *et al*., 2004; Jennings and Bachmann, 2009). CpG-ODN enhance antibody responses in a strongly Th1-biased manner; moreover, DC-specific deletion of TLR signaling in mice indicates that its interaction with DCs and in particular myeloid DCs is a cardinal feature for the antibody-enhancing activity (Hou, Reizis *et al*., 2008). Nevertheless, in primates myeloid DCs are TLR9 negative, suggesting that pDCs are sufficient for the adjuvant effect of CpG-ODN, or that myeloid DCs become indirectly activated in the lymph nodes (Teleshova, Kenney *et al*., 2006).

4.6.3 TLR3 agonists: dsRNA analogues

The discovery that viral dsRNA is a potent activator of innate immunity through TLR3 interaction, has paved the way for extensive studies about synthetic analogues, such as Poly(I:C), as new vaccine adjuvants. Viral or synthetic dsRNA activates TLR3 in endosomes or through cytosolic RNA helicases, such as RIG-I and melanoma differentiation associated gene 5 (MDA5) (Coffman, Sher *et al*., 2010). TLR3 activation in DCs induces IL-12 and type I IFNs and improves MHC class II expression and cross-presentation, whereas stimulation of MDA5, most notably in non-hematopoietic cells, strongly augments the production of type I IFNs, which enhances T and B cell immunity through different mechanisms including activation of DCs, NK cells, and direct effects on T cells (Longhi, Trumpfheller *et al*., 2009). In a murine model of influenza virus infection, intranasal administration of Poly(I:C) with an hemagglutinin-based influenza vaccine induced a strong antibody response against hemagglutinin, whereas vaccination without this TLR agonist displayed little effect. Moreover, the addition of Poly(I:C) protected mice from lethal nasal or pulmonary viral challenge (Ichinohe, Watanabe *et al*., 2005). Other studies in murine models have shown that Poly(I:C) can enhance the efficacy of peptide-based cancer vaccines by promoting tumor-specific T cell responses (Currie, van der Most *et al*., 2008; Salem, Kadima *et al*., 2005; Ammi, De Waele *et al*., 2014).

4.6.4 TLR7 and 8 agonists: imidazoquinolines

TLR7 and 8 are phylogenetically and structurally related (Heil, Hemmi *et al*., 2004), and are localized to the endosomal compartments of human immune cells including DCs, monocytes, macrophages, lymphocytes, Langerhans cells, and NK cells. The interaction between these two TLRs and their cognate ligands activates DCs, by inducing the upregulation of costimulatory molecules and MHC class I and II, and their production of proinflammatory cytokines such as IFNα, TNFα, and IL-12, thus inducing Th1 immune responses. In addition, TLR7 and 8 stimulate both B cells to secrete Abs and produce cytokines, and T cells to proliferate and produce IFN-γ, IL-2, and IL-10 (Steinhagen, Kinjo *et al*., 2011). A new group of TLR7/8 agonists are two synthetic low-molecular weight imidazoquinolines, imiquimod and resiquimod, where the first interacts only with TLR7, while the second acts as ligand for both receptors (Gibson, Lindh *et al*., 2002). Preclinical studies have shown that imidazoquinolines can improve both the magnitude and quality of antigen-specific T cell and Ab responses (Steinhagen, Kinjo *et al*., 2011). For example, in a genetically engineered mouse model, an imiquimod-adjuvanted DNA vaccine encoding for rat HER2/neu significantly delayed the development of spontaneous tumors and reduced their incidence by 65% when compared to DNA vaccine alone (Smorlesi, Papalini *et al*., 2005). Imiquimod is currently approved by FDA as topical medication to treat warts caused by HPV, basal cell carcinoma, and actinic keratosis (Tetif and Serra, 2011; Steinhagen, Kinjo *et al*., 2011).

5. The human epidermal growth factor receptor family

The human epidermal growth factor receptor family consists of four members that belong to the ErbB lineage of proteins, namely HER1 (EGFR, ErbB1), HER2 (HER2, HER2/neu, ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Each of this type I transmembrane protein comprises a heavily glycosylated and disulfide-bonded ectodomain that provides a ligand-binding site, a single transmembrane domain, and a large cytoplasmatic region with a tyrosine kinase activity and multiple phosphorylation sites. The ECD is divided into four regions arranged as a tandem repeat of two types of subdomains: the first and third domains are homologous and have been designated domains I and III or ligand-domain 1 (LD1) and LD2, as they form the ligand-binding site; the second and fourth domains are also homologous and have been designated domains II and IV or cysteine-rich 1 (CR1) and CR2, as nearly 50 conserved cysteines are found in these two regions (Linggi and Carpenter, 2006; Leahy, 2004).

The ErbB receptors recognize 11 different but structurally related growth factors (**Table 4**), which are soluble, small proteins of about 6-10 kDa that share an EGF-like motif of three disulfide

bonds. These ligands are produced through proteolysis of transmembrane precursors at the cell surface by members of the ADAM family of metalloproteases. The activity of these enzymes is a point of fine regulation of ErbB receptors activation, but the molecular regulatory mechanisms are still not clear (Linggi and Carpenter, 2006).

Ligand	Receptor			
	HER1	HER2	HER3	HER4
EGF	+	-	-	-
TGF-α	+	-	-	-
Heparin-binding (HB)-EGF	+	-	-	+
Amphiregulin	+	-	-	-
Betacellulin	+	-	-	+
Epigen	+	-	-	-
Epiregulin	+	-	-	+
Neuregulin-1	-	-	+	+
Neuregulin-2	-	-	+	+
Neuregulin-3	-	-	-	+
Neuregulin-4	-	-	-	+

Table 4. Specificity of interaction between growth factors and ErbB receptors. Several ligands interact with the ErbB family members with different affinity, thus contributing to activate well-defined signaling pathways that in normal conditions lead to cell growth, differentiation, motility, and adhesion. No ligand has been yet identified for HER2 (from Linggi and Carpenter, 2006).

In the absence of a ligand, HER receptors exist as monomers; however, upon ligand binding, they form ten different dimers, which may be homodimers or heterodimers (Baxevanis, Sotiropoulou, *et al*., 2004). In general, in normal cells, HER dimers start a network of different signaling pathways that control normal cell growth, differentiation, motility, adhesion, and apoptosis (Yarden and Sliwkowski, 2001). Moreover, they are implicated in cardiovascular, respiratory, gastrointestinal, integumentary, and nervous system development, since knockout of any of the ErbB receptors is lethal in the mouse embryos (Leahy, 2004). Within a cellular context, the level of activated ErbB receptors is modulated by an increasing number of negative regulators and is positively influenced by other cellular components, such as adhesion molecules (Linggi and Carpenter, 2006). As shown in **Table 4**, HER2 does not bind to known ligands but instead functions as a co-receptor for each of the other three. In fact, even if receptor dimerization

includes both homo- and heterodimerization, ErbB2 is the preferred partner for heterodimerization for all others ErbB receptors. Moreover, because ErbB3 does not contain an active tyrosine kinase, it relies on interaction with ErbB2 for signaling transduction (Linggi and Carpenter, 2006; Roskoski Jr, 2014). The explanation of the tendency of ErbB receptors to preferentially dimerize with HER2 lies in their structure (**Fig. 10**). In fact, in the unbound state, ErbB1, ErbB3, and ErbB4 exist in a tethered intramolecular conformation, in which the dimerization arm of domain II interacts with domain IV. Ligand binding alters this interaction, thus resulting in the exposure of the dimerization arm (Dawson, Bu *et al.*, 2007; Roskoski Jr, 2014). Conversely, HER2 does not adopt an auto-inhibited conformation, being constantly in the "active-like" state in the absence of any ligand. This constantly activated conformation makes HER2 the preferred heterodimerization partner among HER receptors, in particular in cancer, as described later (Leahy, 2004).

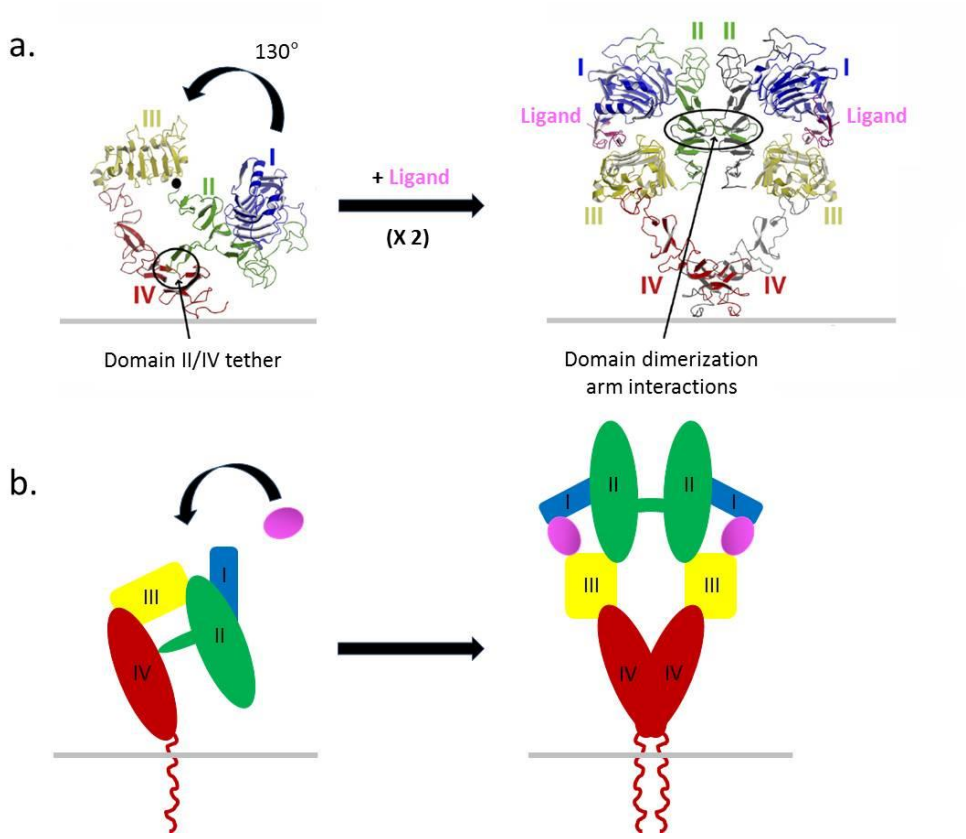


Figure 10. Structures (a) and models (b) of unbound ErbB1, ErbB3, or ErbB4 (left) and ligand-bound ErbB dimers (right). In the unbound state, ErbB receptors adopt a constrained structure in which an extended hairpin loop from domain II (green) binds to a pocket at the C-terminus of domain IV (red). This conformation results in a large separation between domain I (blue) and III (yellow), which together form the ligand binding site. For ligand to bind, the domains II-IV interaction must be broken and a 130° rotation

of the domains I-II pair relative to the domains III-IV pair must occur. This change brings together domains I and III to form a complete binding site, exposing the extended domain II loop that is now free to mediate dimerization (adapted from Dawson, Bu *et al.*, 2007).

5.1 HER2 and neu: role in tumorigenesis and current therapies

HER2 and neu are the human and rodent homologues of an oncogenic growth factor receptor that was identified from human and rodent models respectively, and named independently in the early 1980s, but soon found to be homologues (Slamon, Clark *et al*., 1987). In fact, the aminoacid sequence of the receptor is highly conserved in mammals (Deng, Zheng *et al*., 2014), and the human and rat proteins show a homology of about 89%. While the term *ErbB2* refers to the gene across both human and rodent species, HER2 is used in reference to the human gene and gene product, and neu is used for its rodent counterparts (Moasser, 2007). In this thesis rat neu will be mentioned as rHER2/neu for simplification.

The *neu* oncogene was first described as a transforming oncogene discovered in N-ethyl-N-nitrourea chemically induced neuroblastomas and glioblastomas in rats (Padhy, Shih *et al*., 1982; Drebin, Stern *et al*., 1984), and was shown to transform NIH/3T3 fibroblast cells (Shih, Padhy *et al*., 1981). After cloning of the normal *neu* allele, it was demonstrated that the transforming function in the *neu* oncogene was conferred by a point mutation within the transmembrane domain resulting in a V664E-mutated protein named neuT (Bargmann, Hung *et al*., 1986). This mutation promotes receptor dimerization and enhanced tyrosine kinase activity (Weiner, Liu *et al*., 1989). While rodent *neu* seems to require mutational activation and overexpression for tumorigenicity, human *HER2* appears to hold tumorigenic potential through overexpression alone. In fact, mutations neither within the transmembrane domain nor in the ECD of *HER2* have ever been reported in naturally occurring human cancers, which appear to be always characterized by overexpression of wild-type HER2 (Moasser, 2007). Indeed, overexpression of HER2 protein, through either gene amplification or transcriptional deregulation, occurs approximately in 25-30% of breast and ovarian cancers, 35-45% of pancreatic adenocarcinomas, and up to 90% of colorectal carcinomas, and its overexpression represents a marker of poor prognosis (Slamon, Clark *et al*., 1987; Baxevanis, Sotiropoulou, *et al*., 2004). In fact, breast cancers can present up to 25-50 copies of the *HER2* gene and up to 40- to 100-fold increase in HER2 protein expression, resulting in up to 2 million receptors expressed per cell in malignant tissues, instead of 20,000 to 50,000 molecules per normal cell (Venter, Tuzi *et al*., 1987; Slamon, 2000; Lohrisch and Piccart, 2001). *HER2*-amplified breast cancers show biological features that

distinguish them from other types of breast cancers, which are sensitivity to certain cytotoxic chemotherapeutic agents, resistance to anti-estrogen therapies, and increased propensity to metastasize to the brain (Ross, Fletcher *et al*., 2003; Gabos, Sinha *et al*., 2006).

HER2 overexpression can skew the composition of HER family dimers toward HER2-containing heterodimers and HER2 homodimers, thus leading to deregulation in cell polarity and cell adhesion. For example, HER2 overexpression influences the biological behavior of EGFR. In fact, this receptor is unique among the HER family, as it undergoes endocytic degradation after ligand-mediated activation and homodimerization, in contrast to the other HER members, which undergo endocytic recycling (Baulida, Kraus *et al*., 1996). EGFR-HER2 heterodimers similarly evade endocytic degradation in favor of the recycling pathway and have increased signaling duration and potency, which are even more enhanced when HER2 is overexpressed. Accordingly, HER2-overexpressing cells have significantly prolonged activation of downstream MAPK and c-jun following stimulation with EGFR, thus acquiring proliferative and invasive functions (Karunagaran, Tzahar *et al*., 1996).

The HER2-HER3 heterodimer is the most potent mitogenic combination and is the predominant heterodimer in carcinoma cells (Baxevanis, Sotiropoulou, *et al*., 2004), since it leads to the activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway. Akt lies at the crossroads of multiple signal transduction pathways that regulate numerous critical cellular functions, such as cell proliferation and survival, cell size and response to nutrient availability, glucose metabolism, epithelial-mesenchymal transition and cell invasiveness, genome stability, and angiogenesis (Testa and Bellacosa, 2001).

Moreover, the formation of HER2 homodimers is characteristic of HER2-positive cancer cells. In normal cells, HER2 does not homodimerize; nevertheless, ligand-less HER2 homodimerization spontaneously occurs in cancer cells overexpressing the receptor, due to the large number of molecules present on the cell surface. This fact reflects on high levels of basal tyrosine phosphorylation in human HER2-positive breast and ovarian cancer cells, and the degree of this phosphorylation generally correlates with effects on cellular transformation in a dose-dependent fashion (Reese and Slamon, 1997). Interestingly, in about 15-30% of patients, HER2 undergoes proteolytic cleavage of its ECD (Codony-Servat, Albanell *et al*., 1999), and serum HER2 ECD levels directly correlated with a worse prognosis and insensitivity to endocrine and chemotherapeutic treatments (Molina, Jo *et al*., 1999; Anido, Scaltriti *et al*., 2006).

HER2 status of breast cancers is commonly determined by fluorescence *in situ* hybridization (FISH) to assess *HER2* gene amplification, and immunohistochemistry (IHC) to assess HER2 protein

overexpression, which are both performed at the time of initial diagnosis of primary breast cancer or when a metastatic lesion is biopsied; finally, enzyme-linked immunosorbent assay (ELISA) is used for detection of the biomarker HER2 ECD (Fornier, Seidman *et al*., 2005).

HER2 can represent not only a marker but also a suitable target for cancer therapies. In fact, the receptor constitutes an ideal therapeutic target for breast cancer because: a) the amount of HER2 expressed on cancer cells is much higher than in normal tissues (Press, Cordon-Cardo *et al*., 1990); b) tumors with a high expression of HER2 often show homogeneous and intense IHC staining, thus HER2-specific therapies would successfully target the whole tumor mass (Paik, Hazan *et al*., 1990); c) HER2 is overexpressed both in primary and metastatic lesions, suggesting that HER2-target therapy may be effective in all disease stages (Niehans, Singleton *et al.*, 1993); d) both humoral and cellular immune responses against the self-protein HER2 have been observed without autoimmunity in early-stage breast cancer patients, indicating that it is possible to circumvent tolerance to HER2, and that these immune responses do not seem to recognize normal cells expressing basal levels of this receptor (Disis, Calenoff *et al*., 1994; Disis, Pupa *et al*., 1997; Disis Knutson *et al.*, 2000). Moreover, different T- and B-cell epitopes able to induce immune responses have been identified (Ercolini, Machiels *et al*., 2003; Jacob, Radkevich, *et al*. 2006; Conrad, Gebhard *et al*., 2008). Taken together, these studies suggest that HER2 is a suitable target for cancer immunotherapy, especially for vaccination strategies.

The possibility of blocking HER2-associated cancer progression is confirmed by the documented efficacy of two current therapies employing the humanized mAbs trastuzumab and pertuzumab. Trastuzumab bind the extracellular subdomain IV of HER2, thus impeding homodimerization of HER2, but not its heterodimerization with other members of HER family. The mechanism of action of trastuzumab is not yet well elucidated; however, its antiproliferative and proapoptotic effects are associated with induction of ADCC, CDC, and inhibition of the PI3K pathway and of angiogenesis (Gennari, Menard *et al*., 2004; Vu and Claret, 2012). Current treatment protocols combine trastuzumab with chemotherapy regimens to successfully increase time to tumor progression, and survival of patients. Despite the impressive clinical results with this mAb, positive response is observed in only one third of patients (Kaptain, Tan *et al*., 2001; Bartsch, Wenzel *et al*., 2007), while other patients develop resistance to this mAb (Hynes and Lane, 2005). Finally, the major side effect of trastuzumab is cardiotoxicity, occurring as asymptomatic left ventricular ejection fraction reduction or overt heart failure (Cardinale, Colombo *et al*., 2010).

Through binding to the extracellular domain II of HER2, pertuzumab sterically hinders HER2 dimerization with other receptors of the family, thus effectively blocking ligand–activated

signaling from the HER2/HER1 and HER2/HER3 heterodimers (Sakai, Yokote *et al*., 2007). Since pertuzumab and trastuzumab recognize different sites of HER2 and cause distinct downstream effects, they have displayed a synergistic antitumor effect in combination therapies (Fuentes, Scaltriti *et al*., 2011). However, despite their efficacy in a subset of cancer patients, trastuzumab and pertuzumab are not always sufficient for tumor eradication. Therefore, alternative strategies are currently being investigated, among which HER2-based vaccines seem the most promising in both preventive and therapeutic approaches. In fact, vaccination would stimulate antigen-specific immune responses and immunologic memory, resulting in avoidance of side effects and prevention of relapse, as the risk of recurrence in HER2-positive ovarian and breast cancer patients is extremely high (Gonzalez-Angulo, Litton *et al*., 2009). Finally, HER2-based vaccines could also be exploited in combination therapies with trastuzumab and other therapeutics for the design of better personalized anticancer therapies.

Aim of the project

TLR ligands are emerging as a new class of adjuvants for vaccines, and in particular for cancer vaccines. Indeed, cancer vaccine adjuvants need to be more potent than those for prophylactic vaccines, but the majority of new adjuvants developed resulted too toxic for use in the clinical setting. TLR ligands have already proved to be extremely efficient in promoting the cross-talk between innate and adaptive immune responses, thus playing an important role in antitumor immunity. Of note, TLR ligation can be exploited to reverse peripheral tolerance, and to finely tune the balance between Th1 and Th2 antigen-specific immune responses, thus potentially inducing both humoral and CTL responses. Among TLR agonists, NPs that 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 also are cheap, biocompatible, biodegradable, non-toxic, non-immunogenic, and non-inflammatory. As vaccine adjuvants, they have shown to efficiently activate DCs and confer long-lasting immune responses without any sign of toxicity.

A NP able to interact with two different TLRs, called NPX for patent constraints, has been previously identified in our laboratory as a new promising vaccine adjuvant. The adjuvanticity of NPX was assessed in a variety of model antigens such as ovalbumin (OVA), influenza A virus subtype H5N1 HA, RNase, human growth hormone (hGH), and superoxide dismutase (SOD). Interestingly, NPX boosted strong and sustained immune responses against all the mentioned antigens, provided that it was chemically conjugated to them.

These preliminary data suggested us to employ NPX as adjuvant for anticancer vaccine formulations. Thus, this research project aimed at exploring the potentiality of NPX as new TLR agonist and carrier of cancer immunogens for the induction of safer, more efficient, and durable antitumor immune responses. To this end, the ECD of the rat form of the prototypic oncoantigen HER2/neu was chemically linked to NPX, and the resulting bioconjugate (rHER/neu-NPX) was used for immunization of mice. NPX adjuvanticity profile was evaluated and compared with that of alum, the most common adjuvant for human use.

We focused our attention on the i.m. route of administration, since it allowed us to compare our adjuvant to alum, but also to other commercial adjuvants, whose characteristics are extensively reported in literature. Moreover, since the conjugation with NPX confers solubility in water to the antigen, we investigated and reported preliminary data regarding an innovative route of administration of vaccines, namely the intravenous route, which could have never been exploited before by available adjuvants.

First, we assessed the local safety profile of the adjuvant administered through the i.m. route, by evaluating both muscle integrity and the presence of local inflammatory reactions through histological analysis of tibialis anterior (TA) muscles of immunized mice. Subsequently, BALB/c mice were immunized with different doses and immunization protocols, and the breadth, quality, and persistence of antigen-specific humoral immune responses were monitored for 1 year by ELISA and flow cytometry analysis of mouse sera. Moreover, ELISA test assessment of IgG subclasses and cytokine production of immunized animals allowed us to characterize the T helper profile of immune responses elicited by both adjuvants. Effector immune responses were quantified by using ^{51}Cr release assay. In particular, we evaluated the presence and specificity of CTL responses and the ability of vaccine-induced antibodies to specifically mediate complement-dependent lysis of target cells. Finally, the efficiency of NPX adjuvant in both preventive and therapeutic vaccination settings was assessed through tumor challenge experiments in i.m.-immunized BALB/c mice.

Although mouse models involving tumor cell grafts have contributed to shed light on the efficacy of cancer vaccines, such models do not fully reflect human cancer development that occurs in an immunologically tolerant milieu. Hence, we assessed the ability of rHER2/neu-NPX in breaking the tolerance against the receptor by immunizing i.m. BALB-neuT mice (a transgenic mouse model that constitutively express the receptor), and evaluated whether the immune responses induced can confer protection against spontaneous tumor growth.

Finally, the efficacy of NPX was also assessed in peptide vaccine formulations. We identified and synthesized three potentially immunogenic peptides derived from the ECD of rHER2/neu protein. Peptides were then conjugated to NPX, and the resulting bioconjugates were used for mice immunization. The peptide that gave the best humoral response was conjugated to the PADRE universal Th cell epitope, and NPX adjuvanticity was assessed again in terms of ability to induce antigen-specific humoral responses by ELISA and cytometry analysis.

Materials and Methods

1. Mice

All mouse strains were housed in our Specific Pathogen Free (SPF) animal facility. Procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies (D.L.116/92 and subsequent implementing circulars). The experimental protocols were approved by the local Ethical Committee of Padua University (CEASA). Mouse strains used for this study are reported as follows:

BALB/c mice. Six to eight week-old female BALB/c mice (H-2^d) were purchased from Charles River Laboratories (Calco, Como, Italy).

BALB-neuT mice. Inbred male BALB/c mice carrying the rat ErbB2 transgene under the control of the mouse mammary tumor virus (MMTV) promoter (BALB-neuT; H-2^d) were purchased from Biogem s.c.a.r.l. (Ariano Irpino, Avellino, Italy). Heterozygous female BALB-neuT mice were obtained from the mating of BALB-neuT male mice with wild-type BALB/c females. Progenies were confirmed for the presence of the transgene by PCR, as described below. Eight week-old virgin female BALB-neuT mice were used in our studies.

2. Screening of BALB-neuT mice

Genomic DNA was obtained from young mouse phalanx and ear tissues, and purified using KAPA mouse genotyping kit (Kapa Biosystems, Boston, Massachusetts, United States). Cell lysis, nucleases and proteins degradation, and DNA release were performed by mixing tissues with PCR-grade water, 1 U/µl of KAPA Express Extract Enzyme, and KAPA Express Extract Buffer 10X (both from Kapa Biosystems), and incubating samples in a standard thermocycler for 10 min at 75° C. Inactivation of proteases was performed by incubating the samples for 5 min at 95°C. Finally, samples were centrifuged at high speed for 1 min to pellet debris, and DNA-containing supernatants were recovered.

Genomic DNA samples were then tested by PCR.

The following primers were used:

a) Neu FOR: 5'-GTAACACAGGCAGATGTAGGA-3'

b) Neu REV: 5'-ATCGGTGATGTCGGCGATAT-3'

c) bCasein FOR: 5'-GATGTGCTCCAGGCTAAAGTT-3'

d) bCasein REV: 5'-AGAAACGGAATGTTGTGGAGT-3'

PCR conditions were the following:

1) 95°C 5 min;
2) 94°C 1 min;
3) 58°C 45 sec;
4) 72°C 1 min;

Steps from 2 to 4 were repeated for 35 cycles;

5) 72°C 5 min;
6) 14°C hold.

PCR-amplified DNA samples were then electrophoretically run on a 1.7% agarose gel. The expected fragment lenghts were 525 bp corresponding to *βcasein* gene, and 230 bp corresponding to *neu* gene. Only double-positive virgin female BALB-neuT mice were then used for *in vivo* studies.

3. Tumor cell lines

The following murine tumor cell lines were used:

TUBO is a cloned cell line derived from a lobular carcinoma that arose spontaneously in a female BALB-neuT mouse, thus overexpressing rHER2/neu protein on the cell membrane. This cell line was a generous gift from Professor F. Cavallo, University of Turin, Turin, Italy. TUBO cells were cultured in DMEM (Gibco BRL, Monza, Italy) supplemented with 20% heat-inactivated fetal bovine serum (FBS, Gibco), 10 mM HEPES Buffer, 2 mM L-Glutamine, 100 U/ml Streptomycin, and 100 U/ml Penicillin (all from Lonza, Milan, Italy).

NIH/3T3 is a BALB/c-derived fibroblast cell line. These cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 10 mM HEPES Buffer, 2 mM L-Glutamine, 100 U/ml Streptomycin and 100 U/ml Penicillin, hereafter referred to as DMEM complete medium.

3T3/NKB is a BALB/c NIH/3T3 fibroblast cell line (kindly provided by Dr. W.-Z. Wei, Karmanos Cancer Institute, Detroit, MI, USA) stably cotransfected with rat HER2 and mouse class I H-2K^{d} and B7.1 genes (Wei, Jacob *et al*., 2005). The cell line was maintained in the same medium of TUBO cells, supplemented with 0.8 mg/mL of G418 (Geneticin, Lonza) and 0.8 mg/mL of Zeocin (Invitrogen, San Giuliano Milanese, Italy).

4. rHER2/neu protein and peptides synthesis

The recombinant rHER2/neu ECD (from Methionine 4 to Threonine 656) was purchased from Sino Biological Inc. (Beijing, China) and hereafter referred to as rHER2/neu protein for simplification.

rHER2 sequence was analyzed using bioinformatics tools in collaboration with Professor O. Marin, Department of Biomedical Sciences, University of Padua, Italy. The main goal was to identify the most immunogenic peptides spanning the exposed protein domains. We identified and synthesized 3 main epitopes:

A1: VLDNRDPQDNVAAST

A2: PALVTYNTDTFESMHNPEGRYTFGASC

A3: RNPHQALLHSGNRPEED

To enhance humoral antigen-specific immune responses, we covalently conjugated the last alanine of PADRE universal Th cell epitope (Alexander, Sidney *et al*., 1994; Alexander, del Guercio *et al*., 2004) to the first arginine of A3 sequence. The resulting sequence was:

PADRE-A3: AKFVAAWTLKAAARNPHQALLHSGNRPEED

5. Conjugation of rHER2 and rHER2 peptides to NPX

The conjugation of NPX adjuvant to the C-terminus of rHER2, A1, A2, A3, and PADRE-A3 peptides was performed by Professor G. Pasut, Department of Pharmaceutical Sciences, University of Padua, Italy. 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 always resulted in elimination of LPS contaminant and conferred antigen solubility in water.

6. Hystological assessment of NPX biocompatibility

NPX biocompatibility was compared with that of Imject Alum adjuvant (alum; Thermo Scientific, Milan, Italy), which is a formulation of aluminum hydroxide and magnesium hydroxide, at different time points post injection. To this end, BALB/c mice (three animals per group) were injected i.m. with 5 µg of rHER2/neu conjugated to NPX, or emulsified with alum, or the antigen alone solubilized in phosphate buffered saline (PBS; Sigma-Aldrich, Milan, Italy). This specific concentration was chosen due to antigen solubilization properties. The final volume was 200 µl for TA muscle injection, which is the maximum administrable volume for a single i.m. injection. TA muscles were harvested at 24 h, 72 h, or 1 week after injection; tissue samples were formalin-fixed (1%, Thermo Scientific) for 1 h, paraffin-embedded, and stained with hematoxylin and eosin (H&E) for histologic evaluation. The H&E staining was performed in collaboration with Melanoma and Sarcoma Diagnostics Section, Veneto Institute of Oncology IOV-IRCCS, Padua, Italy.

7. Mice immunization protocols and serum collection

BALB/c mice were immunized i.m. with both 10 (5 μg per muscle) or 1 μg of rHER2/neu-NPX or the same amounts of protein emulsified in alum, or the protein alone in PBS as controls, and i.v. with 10 or 1 μg of the bioconjugate or free protein in PBS (six mice per group).

To investigate the ability of NPX to induce immune responses against rHER2 peptides, BALB/c mice were immunized i.m. with 30 μg of A1, A2, A3, or PADRE-A3 peptides, or 100 μg of A3 peptide, which were all administered either conjugated to NPX, or emulsified with alum, or alone in PBS (three mice per group).

To assess the ability of NPX vaccination to break immune tolerance against rHER2/neu, BALB-neuT mice were immunized i.m. with 10 μg of rHER2-NPX or the same amount of protein adsorbed to alum (four mice per group), before the appearance of palpable tumors.

For both mouse strains, the immunization schedule consisted in a priming step at day 0, followed by two boosts at days 14 and 21. Mice that were injected i.v. with 10 μg of bioconjugate or free protein received only the priming step. The relative sera were collected at day 0 as basal control, immediately before every subsequent immunization, and thereafter every 30 days up to six months, with a final time point at 1 year.

8. Serum IgG and IgG subclasses titration: ELISA test

Individual sera were titrated for protein or peptide-specific IgG and IgG subclasses (IgG1, IgG2a, and IgG2b) content by ELISA test. Three μg/ml of rHER2/neu protein or 10 μg/ml of A1, A2, or A3 peptides were coated overnight at 4°C on a 96-well plate half area (Corning Life Sciences, NY, USA) in 0.005 M sodium carbonate/bicarbonate buffer pH 9.6 (Sigma-Aldrich). The plate was washed 5 times with 100 μl/well PBS, and incubated for 2 h at room temperature (RT) with 100 μl/well of milk 2% (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) diluted in PBS. The plate was washed 5 times with 100 μl /well PBS and incubated for 1 h at RT with 25 μl/well of sequential dilutions of mouse sera in milk 2%, starting from 1:50 to 1:6400. Negative controls were milk 2% alone and sera at day 0 diluted 1:50 in milk 2%. The plate was washed 5 times with 100 μl/well PBS and incubated for 1 h at RT with secondary HRP-conjugated goat polyclonal anti-mouse total IgG (IgG-heavy and light chain antibody), IgG1, IgG2a, or IgG2b antibodies (all from Bethyl Laboratories, Inc., Montgomery, TX, USA), at the final concentration of 0.02 μg/ml in PBS-0.05% Tween 20 (Sigma-Aldrich). The plate was washed 5 times with 100 μl/well PBS, and 60 μl/well of substrate OPD solution (o-Phenylenediamine dihydrochloride, Sigma-Aldrich) were added for 10

min at RT in the dark. Reaction was stopped with 30 µl/well of HCl 3N (Carlo Erba, Milan, Italy), and the absorbance was read at 490 nm using VictorX4 Multilabel Plate Reader (PerkinElmer, Boston, MA, USA).

Quantification of IgG and IgG subclasses content was assessed by performing titration curves. Briefly, 5 µg/ml of goat anti-mouse polyvalent immunoglobulins (Sigma-Aldrich) were coated overnight at 4°C on a 96-well plate half area in 0.005 M sodium carbonate/bicarbonate buffer pH 9.6 (Sigma-Aldrich). The plate was washed 5 times with 100 µl/well PBS, blocked for 2 h with milk 2%, and washed again with 100 µl/well PBS. The plate was then incubated for 1 h at RT with 25 µl/well of sequential dilutions (from 1:25600 to 1:8192000) of IgG from mouse serum (Sigma-Aldrich) or previously purified and quantified IgG1 (2.3 mg/ml), IgG2a (5.3 mg/ml), and IgG2b (1 mg/ml) 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.

9. Cell staining and flow cytometry analysis

The same sera used in the ELISA assays were analyzed for their ability to specifically recognize rHER2/neu in its native conformation; $2x10^5$ rHER2/neu-positive TUBO and 3T3/NKB cells, and rHER2-negative NIH/3T3 cells were labeled for 30 min in ice with 50 µl of 0, 14, 30, 90, 150, or 360-day pooled sera, at a final dilution of 1:100 in staining buffer. As positive control, cells were incubated with mouse anti-rHER2/neu mAb (Ab4, clone 7.16.4, Calbiochem, San Diego, CA, USA), at a final concentration of 0.16 µg/ml in buffer for 30 min in ice. Cells were then washed and labeled with secondary APC goat anti-mouse IgG (clone poly4053, BioLegend, London, UK), 1 µl in 50 µl of buffer for 30 min in ice in the dark. Finally, cells were washed and resuspended in 250 µl of PBS. Cells were then analyzed using FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (TreeStar Inc., Olten, Switzerland), and mean fluorescence intensity (MFI) was calculated. A standard curve for rHER2/neu binding was generated as performed by Piechocki *et al*. (Piechocki, Pilon *et al*., 2002) with few modifications. Briefly, 3T3/NKB cells were incubated with scalar concentrations of anti-rHER2/neu Ab4 mAb, starting from 1:1000 to 1:20000 in 50 µl of buffer for 30 min in ice. Cells were then treated as described above. Results were expressed as MFI and the correlation between MFI and IgG concentration was analyzed by linear regression analysis.

10. Complement-Dependent Cytotoxicity

Target cells ($1x10^6$ 3T3/NKB and NIH/3T3 cells) were labeled with 100 µCi $Na_2{}^{51}CrO_4$ (DuPont, Boston, MA, USA) for 1 h at 37°C. Cells were then washed twice and resuspended in DMEM 3% FBS. Radiolabeled cells were seeded at $2x10^3$ cells/well in triplicates in round-bottom 96-well plate (Sarstedt, Verona, Italy), and incubated for 1.5 h at 4°C with individual sera diluted 1:50 in DMEM 3% FBS, in a final volume of 50 µl/well. After washing with 100 µl/well of PBS, supernatants were discarded and cells were resuspended in 200 µl/well of DMEM 20% Rabbit Complement (Low-Tox-H Rabbit Complement, Cedarlane, Burlington, Canada), for 1.5 hour at 37°C. As negative control (spontaneous release), cells were incubated with DMEM 3% FBS and then with rabbit complement, while for positive control (maximum release) cells were treated with 200 µl/well of Triton 5% (Sigma-Aldrich). At the end of incubation, 30 µl of supernatants were transferred in a 96-well solid scintillator coated plate (LumaPlate-96, PerkinElmer) and radioactivity was evaluated using a γ-ray counter (TopCount NXT, PerkinElmer). The cytotoxicity index was evaluated as follows:

$$C.I. = 100\,x\,\frac{\text{cpm test - cpm spontaneou s release}}{\text{cpm maximum release - cpm spontaneou s release}}$$

11. Cytokine production

To define the Th1/Th2 profile induced by NPX-vaccination, BALB/c mice were vaccinated i.m. with 10 µg of rHER2/neu-NPX or rHER2/neu emulsified with alum as control as described above, 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 10^5 syngeneic irradiated (60 Gy) TUBO, 3T3/NKB, and NIH/3T3 cells, or 1 µg/ml of rHER2/neu 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 24 and 72 h. Samples were analyzed for their cytokine content (IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12p70, IL-17,and TNFα) by ELISA test using Ciraplex Mouse Cytokine 1 8-plex array kit (Aushon BioSystems, Inc., MA, USA), according to the manufacturer's instructions.

12. Prophylactic and therapeutic models of TUBO challenge

For the prophylactic model of vaccination, BALB/c mice (six animals per group) were vaccinated i.m. as described above, and then challenged at day 30 with $1x10^5$ TUBO cells injected into the mammary fat pad, to compare the protective activity of rHER2/neu-NPX vaccination strategy with that induced by the protein emulsified in alum. Control group was represented by mice that were not vaccinated and received only TUBO injection. Mice were observed 3 times per week to monitor tumor growth by measuring maximum and minimum diameter. Tumor mass was calculated applying the formula:

$$Tmass = \frac{d^2 \times D}{2},$$

where d and D are minimum and maximum diameter, respectively.

To determine whether the immune response deriving from anti-rHER2/neu vaccination resulted in memory induction, mice that survived from tumor challenge were evaluated for long-term protection by rechallenge with $1x10^6$ TUBO cells about 3 months after the first challenge. Control group was represented by mice that received only TUBO injection. Mice were observed 3 times per week and tumor mass was calculated as described.

For the therapeutic model of vaccination, BALB/c mice (six animals per group) were first injected into the mammary fat pad with $1x10^5$ TUBO cells, and when all mice had an established tumor of ~3 mm in diameter, they were vaccinated i.m. with 10 or 1 µg of rHER2/neu conjugated with NPX or emulsified with alum at day 12 (priming), and then at days 19 and 26 (boosts). Control group was represented by mice that received only TUBO injection.

In these experiments, measurable masses >2 mm diameter were regarded as tumors. When tumor volumes were >400 mm^3 (for the preventive model of TUBO challenge and rechallenge), or >1000 mm^3 (for the therapeutic model of TUBO challenge), or when skin ulceration occurred, mice were sacrificed by CO_2 inhalation, according to the guidelines of the UK Coordinating Committee Cancer Research (UKCCCR) (Cancer Metastasis 1989, "UKCCCR guidelines to the welfare of animals in experimental neoplasia").

13. Mixed Leukocyte Tumor Cell cultures (MLTC)

Spleens of BALB/c mice immunized i.m. with 10 µg of rHER2/neu-NPX or rHER2/neu emulsified with alum as described above, and spleens of TUBO-challenged/rechallenged mice in the

prophylactic vaccination model, were collected at day 30 or at sacrifice, respectively. MLTC cultures were set up by *in vitro* restimulation of $25x10^6$ splenocytes with 10^6 syngeneic irradiated (60 Gy) rHER2/neu-positive TUBO and 3T3/NKB cells, or rHER2/neu-negative NIH/3T3 cells as control. Cell cultures were maintained in DMEM complete medium, in 25-cm^2 tissue culture flasks (Falcon, Becton Dickinson) for 5 days at 37°C, 5% CO_2, and finally tested for lytic activity in a ^{51}Cr-release assay.

14. Cytotoxicity assay

The cytotoxic activity of MLTC was assessed in a 4h ^{51}Cr-release assay after 5 days of culture. Briefly, TUBO, 3T3/NKB, and NIH/3T3 cells (target cells) were labeled for 1 h at 37°C with 100 µCi $Na_2{}^{51}CrO_4$, 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) 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 Triton 5%. 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) and radioactivity was evaluated using TopCount NXT γ-ray counter (PerkinElmer). The cytotoxicity index was evaluated as previously described.

15. Prevention of spontaneous tumor growth in BALB-neuT mice

To assess the ability of NPX vaccination to prevent or delay the occurrence of spontaneous HER2-positive mammary neoplasms, eight week-old virgin female BALB-neuT mice were immunized as described above, while control group was represented by BALB-neuT that did not received immunization. Appearance of spontaneous tumors was monitored by manual inspection of mammary pads 3 times per week. Data are reported as tumor multiplicity (cumulative number of tumors per number of mice in each group). Measurable/palpable masses >2 mm in diameter were regarded as tumors. Also in this experiment, when tumor volumes were >1000 mm^3 or when skin ulceration occurred, mice were sacrificed by CO_2 inhalation, according to the guidelines of the UKCCCR.

16. Statistical analysis

For the kinetics data, the ANOVA test for repeated measurements was performed to determine differences between the tumor growth in control and vaccinated groups. $P<0.05$ was considered to be statistically significant. Kaplan–Meier product-limit method was carried out to estimate the survival curves, and comparison of survival between groups was performed using the log-rank test. Statistical differences between percentage of CDC-mediated cell lysis in ^{51}Cr-release assays, antibody titers, and cytokines production evaluated by ELISA test were calculated with the Student *t* test for independent samples. All statistical analysis were performed using MedCalc, version 12.1.0.

Results

1. Hystological assessment of NPX biocompatibility

All effective vaccines stimulate the innate immune system to produce cytokines and chemokines for the development of adaptive immune responses. These inflammatory reactions are usually local and transient, but, in some cases, severe side effects can occur, with uncontrolled release of tissue damage-associated danger signals. Therefore, the investigation of local reactions following vaccine administration is essential to understand the safety profile of vaccine adjuvants. To this purpose, we immunized BALB/c mice with a single i.m. injection of rHER2/neu recombinant protein conjugated to NPX, emulsified with alum, or alone as negative control. TA muscles were harvested at different time points thereafter, and the presence of local inflammatory reactions and muscle integrity were evaluated by H&E staining (**Fig. 1**).

As expected (Kashiwagi, Maeda *et al.*, 2014), in mice immunized with alum a typical local inflammatory reaction at the site of injection occurred, with a massive recruitment of neutrophils and monocytes, which were already detectable 24 h after immunization. The number of inflammatory cells increased over time, and at 72 h macrophages were the most represented immune cells, characterized by ballooned cytoplasm with peripherally localized nuclei. Aggregates of macrophages were even more evident 1 week after immunization. Conversely, neither NPX nor the protein alone induced any histological changes indicative of inflammation; as a result, muscle integrity was totally preserved. Therefore, NPX appeared to be extremely safe, and to act differently from classical immunological adjuvants that trigger strong inflammatory responses at the site of injection.

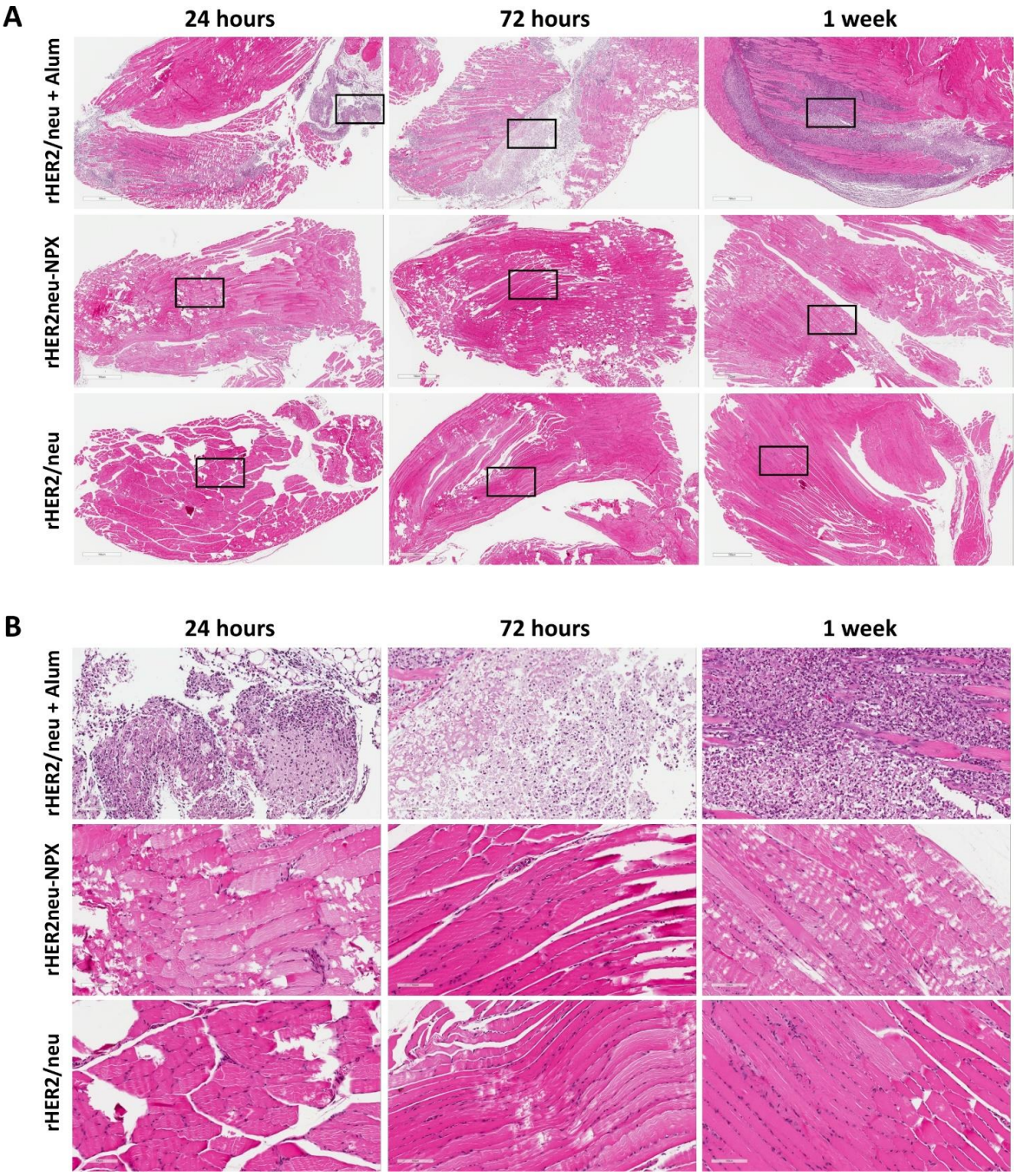


Figure 1. Histological analyses of tibialis anterior muscles from vaccinated BALB/c mice. BALB/c mice were immunized i.m. with 5 µg of rHER2/neu-NPX, rHER2/neu emulsified with alum, or the protein alone. Muscles were harvested at 24 h, 72 h, or 1 week after injection and stained with H&E for histologic evaluation. Histological analyses are reported at (A) 3X, and (B) 20X magnification of the framed areas in A.

2. Assessment of humoral responses: serum IgG and IgG subclasses titration

To compare the breadth and quality of antigen-specific humoral immune responses induced by NPX- or alum-adjuvanted vaccination, we immunized i.m. BALB/c mice at days 0, 14, and 21 with 10 or 1 μg of rHER2/neu emulsified with alum or conjugated to NPX. Since rHER2/neu is a xenogeneic protein in normal mice, albeit only 6% of the amino acid residues differ from the mouse ErbB2, additional control mice were immunized with the same amounts of protein alone, according to the same protocol.

As regards the i.v. route of immunization, preliminary data obtained in our laboratory and involving the OVA model antigen, showed that NPX is extremely efficient in inducing high antigen-specific IgG titers, thus requiring lower number of vaccination doses or lower amounts of antigen per dose. In particular, i.v. administration of a single dose of 10 μg OVA-NPX demonstrated to be sufficient to induce strong humoral responses. Encouraged by these results, we immunized further BALB/c mice i.v. at day 0, 14, 21 with 1 μg of rHER2/neu-NPX or with a single dose of 10 μg of the bioconjugate at day 0. As the i.v. route cannot be exploited by commercially available adjuvants, control groups were represented by BALB/c mice immunized with the same amounts of rHER2/neu alone.

Adjuvanticity of NPX and alum was evaluated in terms of quantity, quality and persistence of the humoral immune responses induced. For this purpose, other than total IgG, IgG1, IgG2a, and IgG2b subclass titers were evaluated and monitored over time. This allowed us to indirectly estimate the involvement of Th cells responsible for isotype switching, and the balance between Th1 and Th2 responses.

The immunization with protein alone did not induce any detectable response, while NPX for both routes of administration and alum induced strong anti-rHER2/neu humoral responses, which persisted over time being still detectable at 1 year after priming (**Fig. 2**). Notably, mice immunized with rHER2/neu-NPX disclosed increased IgG titers in comparison to control groups. In particular, for both routes of administration, these humoral responses were already detectable after priming at day 14, except for mice immunized i.v. with 1 μg of the bioconjugate whose IgG production was detectable after 1 boost (day 21). Conversely, alum-vaccinated mice always required 1 or 2 boosts of 10 or 1 μg of rHER2/neu, respectively, to induce any detectable response. Moreover, while both adjuvants elicited strong IgG1 responses, the bioconjugate clearly disclosed higher IgG2a and IgG2b titers, which were also detectable for the i.v. route of administration. Taken

together, these data demonstrate the superiority of NPX adjuvanticity over alum, since NPX-based vaccinations require not only lower number of doses but also lower amounts of antigen per dose to induce detectable humoral responses.

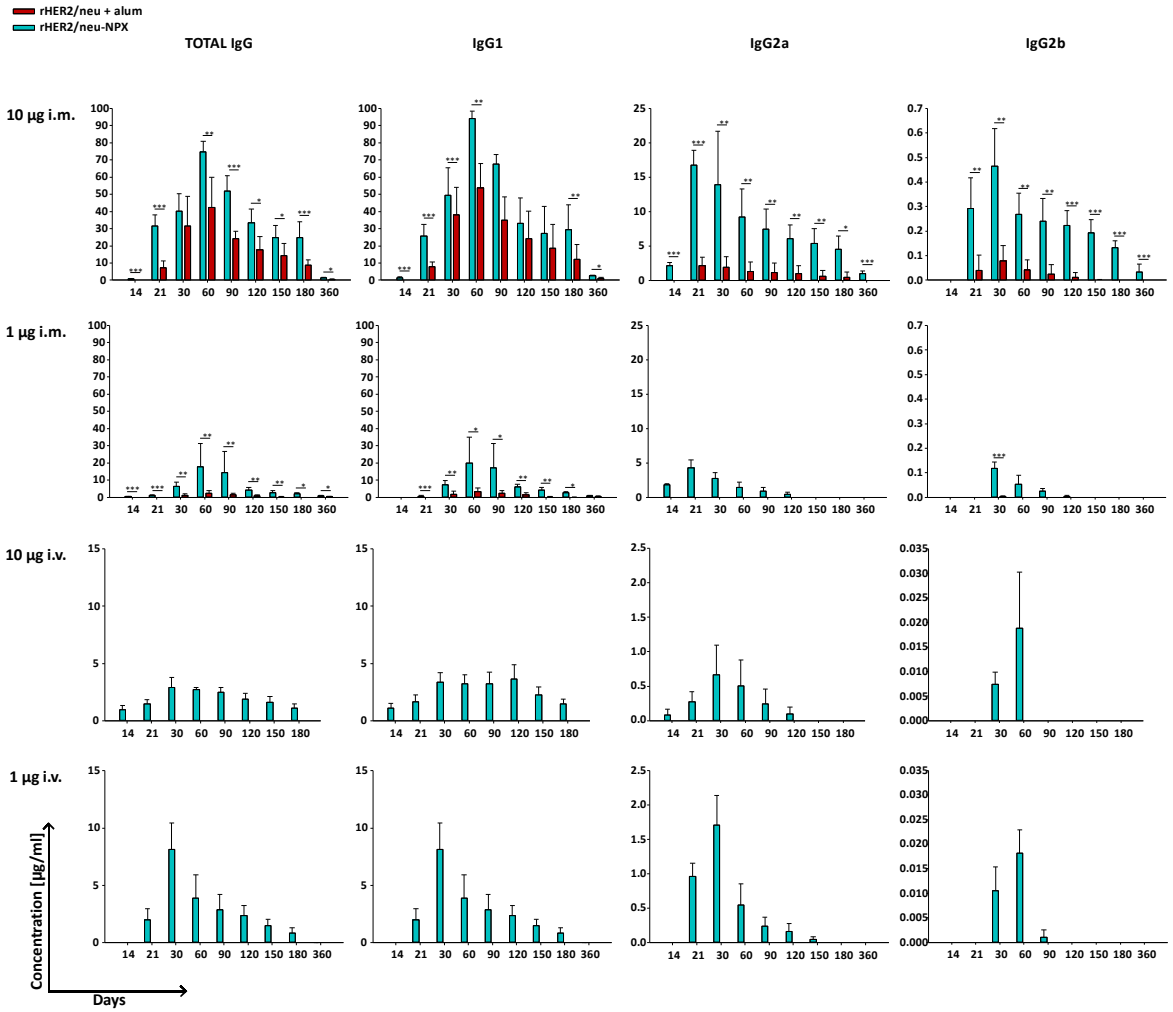


Figure 2. Total IgG and IgG subclass contents in sera from vaccinated BALB/c mice. BALB/c mice were immunized i.m. with 10 or 1 µg rHER2/neu conjugated to NPX or emulsified with alum, or i.v. with 10 µg or 1 µg of the bioconjugate or the protein alone (6 mice/group). Immunization protocol consisted in a prime dose at day 0, followed by 2 boosts at days 14 and 21, except for i.v. administration of 10 µg of the bioconjugate, which consisted in the priming only. Sera were collected at day 0, as basal control, immediately before every subsequent immunization, and thereafter every 30 days up to six months, with a final time point at 1 year (time point not available for 10 µg i.v. immunization). IgG and IgG subclass content was quantified by ELISA test. Where concentration values are not reported, IgG and IgG subclasses content was below the detection threshold level for this method. * P<0.05; ** P<0.005; *** P<0.001, Student's *t* test for independent samples.

The induction of these various IgG subclasses is suggestive of the Th cell polarization promoted by either adjuvants. In particular, in mice immunized with alum IgG1 subclass dominated the immune response as clearly indicated by the IgG2a/IgG1 ratio (**Table 1**), thus confirming that alum induced a Th2-skewed immune response. Conversely, rHER2/neu-NPX stimulated a more balanced IgG2a/IgG1 humoral response. In particular, mice immunized i.m. with the bioconjugate disclosed a more balanced Th1/Th2 immune response, while i.v. administration of the bioconjugate seemed to preferentially boost Th2-skewed responses.

<table>
<tr><th rowspan="3">Time point of serum collection</th><th colspan="2">IgG2a : IgG1 ratio</th><th colspan="2">IgG2a : IgG1 ratio</th><th colspan="2">IgG2a : IgG1 ratio</th></tr>
<tr><td colspan="2">10 µg i.m.</td><td colspan="2">1 µg i.m.</td><td>10 µg i.v.</td><td>1 µg i.v.</td></tr>
<tr><td>rHER2/neu-NPX</td><td>rHER2/neu+alum</td><td>rHER2/neu-NPX</td><td>rHER2/neu+alum</td><td>rHER2/neu-NPX</td><td>rHER2/neu-NPX</td></tr>
<tr><td>14</td><td>1.40 : 1</td><td>/</td><td>IgG2a only</td><td>/</td><td>1 : 13.42</td><td>/</td></tr>
<tr><td>21</td><td>1 : 1.53</td><td>1 : 3.65</td><td>9.55 : 1</td><td>/</td><td>1 : 6.18</td><td>1 : 2.07</td></tr>
<tr><td>30</td><td>1 : 3.55</td><td>1 : 19.84</td><td>1 : 2.73</td><td rowspan="6">IgG1 only</td><td>1 : 5.02</td><td>1 : 5.18</td></tr>
<tr><td>60</td><td>1: 10.24</td><td>1 : 43.38</td><td>1 : 13.97</td><td>1 : 6.42</td><td>1 : 8.75</td></tr>
<tr><td>90</td><td>1 : 7.36</td><td>1 : 28.18</td><td>1 : 19.17</td><td>1 : 13.57</td><td>1 : 18.12</td></tr>
<tr><td>120</td><td>1 : 5.48</td><td>1 : 25.26</td><td>1 : 14.32</td><td>1 : 37.38</td><td>1 : 21.12</td></tr>
<tr><td>150</td><td>1 : 5.11</td><td>1 : 34.44</td><td rowspan="3">IgG1 only</td><td rowspan="2">IgG1 only</td><td>1 : 41.78</td></tr>
<tr><td>180</td><td>1 : 6.47</td><td>1 : 27.81</td><td rowspan="2">IgG1 only</td></tr>
<tr><td>360</td><td>1 : 2.61</td><td>IgG1 only</td><td>not available</td></tr>
</table>

Table 1. IgG2a/IgG1 ratio in sera from vaccinated BALB/c mice. The IgG2a/IgG1 ratio was calculated from the mean value of IgG1 and IgG2a content (µg/ml) of sera collected at different time points from BALB/c mice immunized i.m. with 10 µg or 1 µg of rHER2/neu conjugated to NPX or emulsified with alum, or i.v. with 10 µg or 1 µg of the bioconjugate (6 mice/group).

The induction of strong antigen-specific humoral responses is a great challenge for peptide vaccination. In general, short peptides are poorly immunogenic and commercially available adjuvants often failed to induce any detectable immune response. In collaboration with Prof. O. Marin, we identified and synthesized three potentially immunogenic peptides from the ECD of rHER2/neu protein, named A1, A2, and A3. We therefore immunized i.m. BALB/c mice according to the standard schedule (priming and two boosts), with 30 µg of these peptides conjugated to NPX or emulsified with alum, and evaluated IgG titers. A1 and A2 peptides did not induce any detectable humoral response (data not shown); conversely, mice immunized with A3-NPX

developed peptide-specific IgG, whereas alum completely failed to induce any detectable response (**Fig. 3, left panel**).

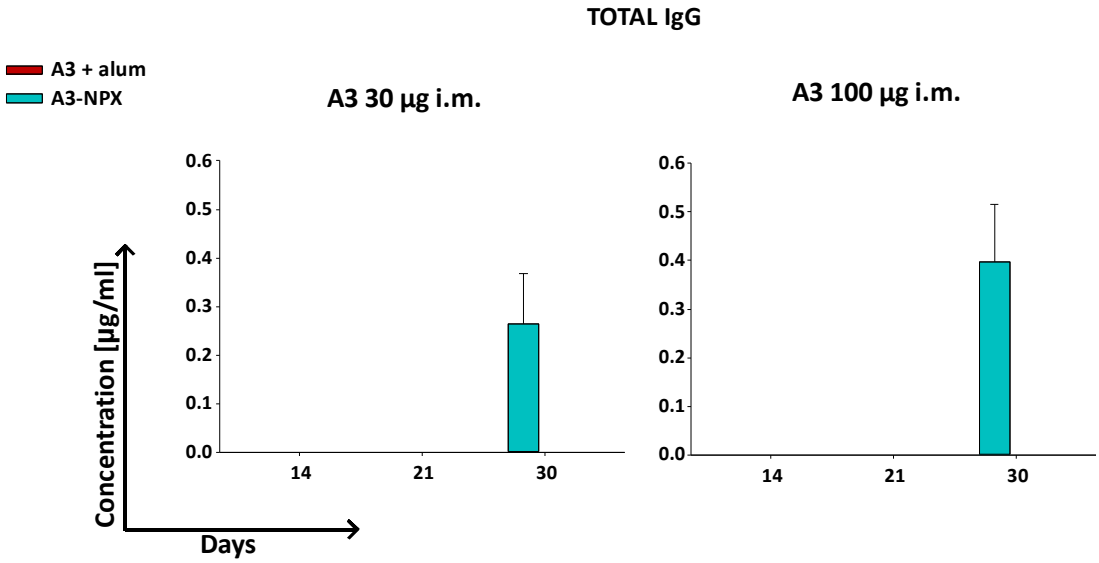


Figure 3. IgG content of sera from BALB/c mice immunized i.m. with A3 peptide conjugated to NPX. BALB/c mice were immunized i.m. at day 0, 14, and 21 with 30 µg (left panel) or 100 µg (right panel) of A3 peptide conjugated to NPX or emulsified with alum (3 mice/group). Sera were collected at day 0, as basal control, before every subsequent immunization, and at day 30. IgG content was calculated by ELISA test. Where concentration values are not reported, IgG content was below the detection threshold level for this method.

To strengthen A3-specific humoral response, mice were immunized i.m. with 100 µg of A3-NPX or A3 emulsified with alum. Again, only mice immunized with the bioconjugate produced detectable amounts of IgG (**Fig. 3, right panel**). Since the conjugation of small peptides to the PADRE universal Th cell epitope proved to enhance humoral antigen-specific immune responses in mice (Alexander, Sidney *et al*., 1994; Alexander, del Guercio *et al*., 2004), we covalently conjugated the last alanine of PADRE peptide to the first arginine of A3 sequence. Thirty µg of the resulting PADRE-A3 peptide conjugated to NPX or emulsified with alum, were then administered i.m. to BALB/c mice according to our standard vaccination schedule. The bioconjugate provided a strong boost for humoral response for all IgG subclasses, while mice immunized with alum developed a very low antibody response (**Fig. 4**).

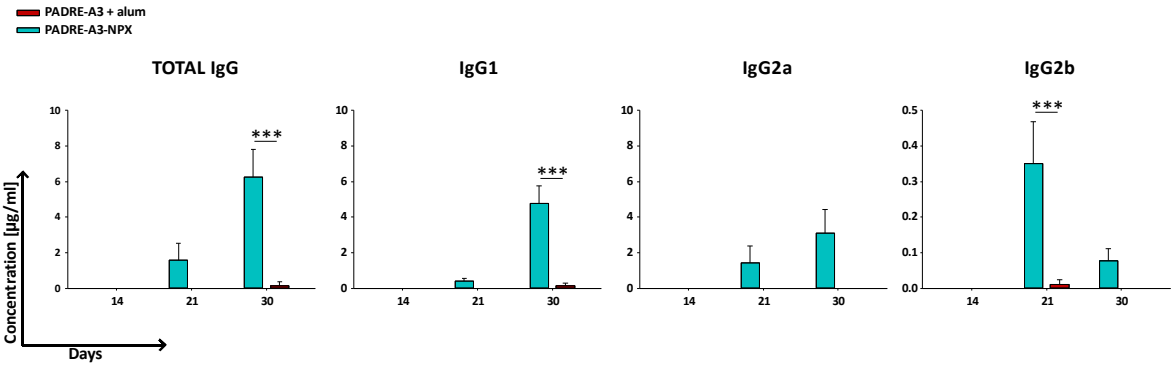


Figure 4. Total IgG and IgG subclass content of sera from BALB/c mice immunized i.m. with PADRE-A3 conjugated to NPX. BALB/c mice were immunized i.m. at day 0, 14, and 21 with 30 µg of PADRE-A3 conjugated to NPX or emulsified with alum (3 mice/group). Sera were collected at day 0, as basal control, before every subsequent immunization, and at day 30. IgG and IgG subclass content was calculated by ELISA test. Where concentration values are not reported, immunoglobulins content was below the detection threshold level for this method**.** *** P<0.001, Student's *t* test for independent samples.

As previously described, also in this case the different IgG subclasses elicited mirrored the differential Th cell polarization promoted by alum and NPX adjuvants, with a complete Th2-skewed immune response induced in alum-vaccinated mice (**Table 2**). Conversely, in mice immunized with PADRE-A3-NPX the presence of both Th1 and Th2 immune responses was observed.

Time point of serum collection	IgG2a : IgG1 ratio	
	PADRE-A3 30 µg i.m.	
	PADRE-A3-NPX	PADRE-A3+alum
14	/	/
21	3.52 : 1	/
30	1 : 1.53	IgG1 only

Table 2. IgG2a/IgG1 ratio of sera from BALB/c mice immunized i.m. with PADRE-A3 conjugated to NPX. The IgG2a/IgG1 ratio was calculated from the mean value of IgG2a and IgG1 content (µg/ml) of sera from BALB/c mice immunized i.m. with 30 µg of PADRE-A3 conjugated to NPX or emulsified with alum (3 mice/group).

Another great challenge for the generation of efficient cancer vaccines is represented by the need to break tolerance against self tumor-associated antigens. In this regard, transgenic BALB-neuT

mice are tolerant to the rHER2/neu protein because they express the transgene in the thymus early in life. To assess the ability of NPX to break immune tolerance against rHER2/neu, female BALB-neuT mice were immunized i.m. with 10 µg of rHER2/neu protein conjugated to NPX or emulsified with alum, as this dosage proved to elicit the highest IgG titers in BALB/c mice. To verify if Abs production occurred spontaneously in this mouse strain, sera from non-immunized animals were collected at the same time points of vaccinated groups, and evaluated for their IgG content. Both vaccine adjuvants succeeded in breaking tolerance against rHER2/neu and eliciting strong antibody titers (**Fig. 5**), while non-vaccinated BALB-neuT mice did not mount any detectable spontaneous humoral response (data not shown). Notably, NPX-vaccinated animals exhibited IgG titers that were about two-fold higher than those observed with alum.

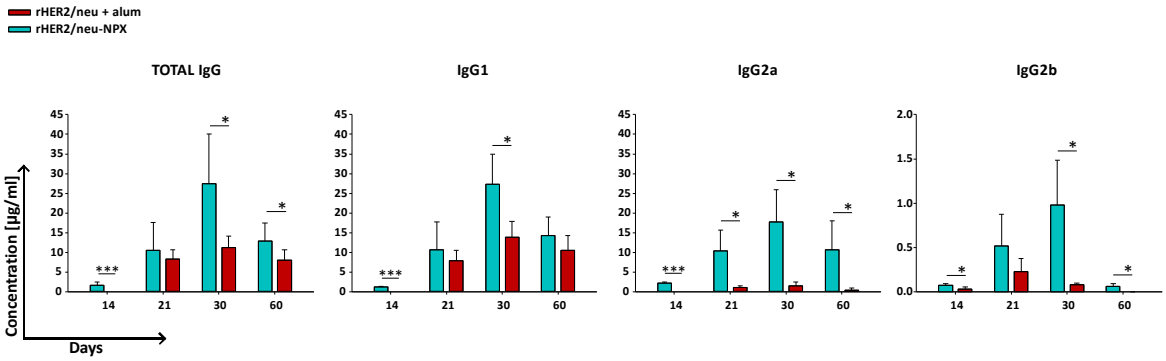


Figure 5. Total IgG and IgG subclass content of sera from vaccinated BALB-neuT mice. BALB-neuT mice were immunized i.m. with 10 µg of rHER2/neu conjugated to NPX or emulsified with alum (4 mice/group). Immunization protocol consisted in a priming at day 0, followed by 2 boosts at days 14 and 21. Sera were collected at day 0, as basal control, before every subsequent immunization, and at days 30 and 60. Titration of IgG and IgG subclasses was performed by ELISA test. * P<0.05; *** P<0.001, Student's *t* test for independent samples.

All three IgG subclasses were produced by vaccinated groups albeit to a different extent, but interestingly in transgenic mice NPX vaccination resulted in a more balanced Th1/Th2 ratio when compared to immune responses in BALB/c mice, whereas alum-vaccinated group still displayed a Th2-skewed immune response, even though less pronounced (**Table 3**).

Time point of serum collection	IgG2a : IgG1 ratio	
	rHER2/neu 10 µg i.m.	
	rHER2/neu-NPX	rHER2/neu+alum
14	1.74 : 1	/
21	1 : 1.01	1 : 6.85
30	1 : 1.54	1 : 8.59
60	1 : 1.34	1 : 22.59

Table 3. IgG2a/IgG1 ratio from sera collected from vaccinated BALB-neuT mice. IgG2a/IgG1 ratio was calculated from the mean value of IgG2a and IgG1 content (µg/ml) of sera collected at different time points from BALB-neuT mice immunized i.m. with 10 µg of rHER2/neu conjugated to NPX or emulsified with alum (4 mice/group).

3. Antigen-specific IgG binding: flow cytometry analysis

Since protein- and peptide-based vaccinations induce antibodies that have seldom showed high affinity for the native protein (Dakappagari, Douglas *et al.*, 2000), we evaluated by flow cytometry the ability of the same sera analyzed by ELISA test to recognize and bind rHER2/neu in its native conformation. First, the expression of rHER2/neu on TUBO and 3T3/NKB cells (positive controls) and on NIH/3T3 cells (negative control) was assessed by staining with the commercial anti-rHER2/neu Ab4 mAb (**Fig. 6**), and then IgG sera were used to stain the same panel of cells (**Fig. 7**).

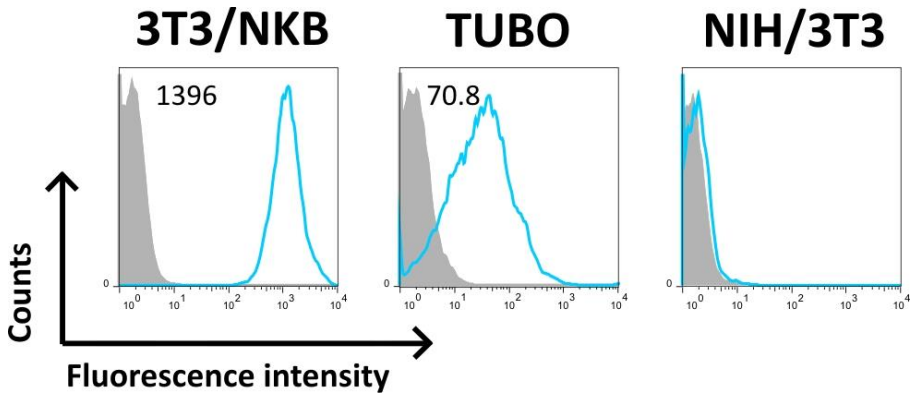


Figure 6. Expression of rHER2/neu receptor on 3T3/NKB, TUBO and NIH/3T3 cell lines. rHER2/neu-positive (3T3/NKB and TUBO) and negative (NIH/3T3) cell lines were stained with the commercial anti-rHER2/neu Ab4 mAb (light blue curves, rHER2/neu staining; grey plot, isotype control). Numbers reported indicate MFI of Ab4 staining.

As expected on the basis of ELISA analysis, sera from mice immunized with protein alone did not stain rHER2/neu-expressing cell lines. Conversely, mice immunized with alum or NPX from both routes of administration disclosed strong and persistent humoral responses, still able to specifically recognize rHER2/neu at 1 year after priming (**Fig. 7**).

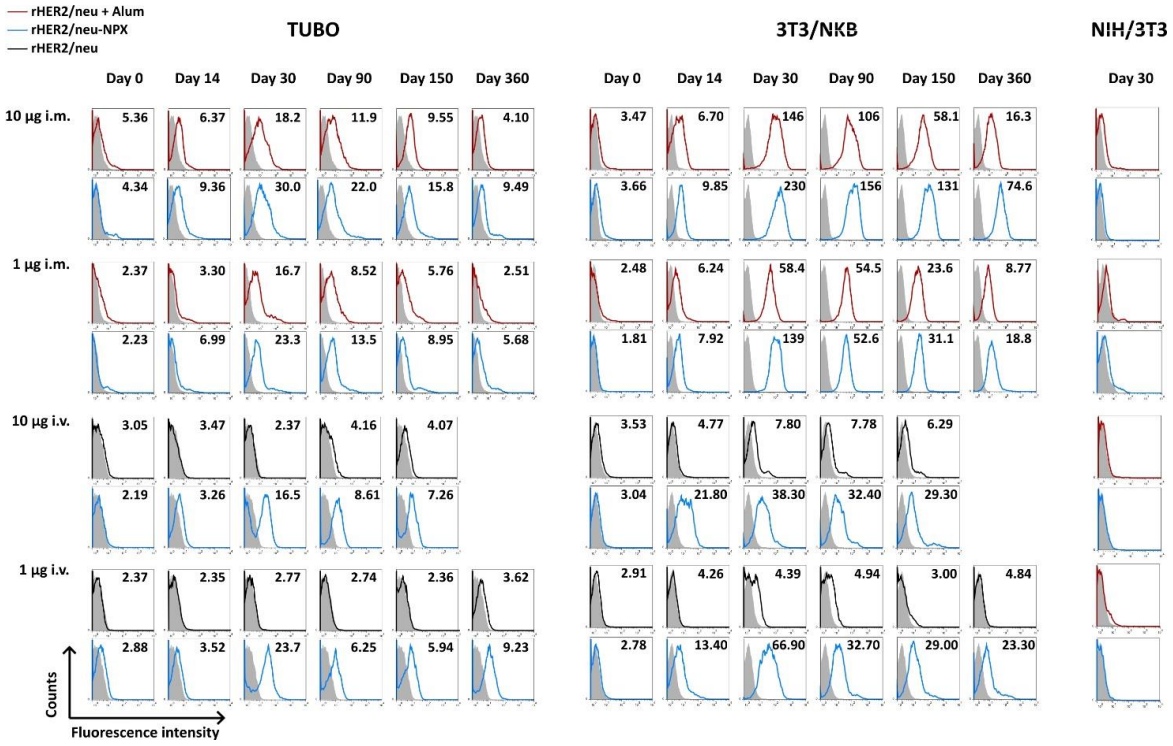
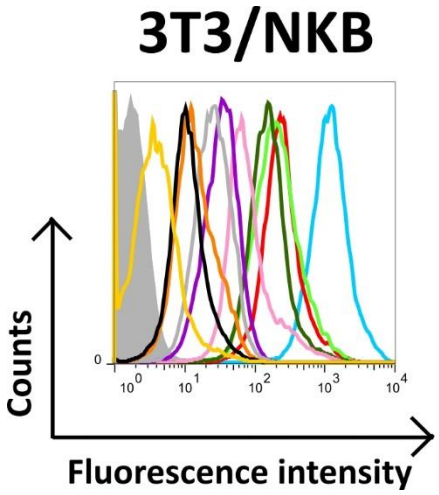


Figure 7. Flow cytometry analysis of rHER2/neu-positive and negative cell lines, stained with pooled sera from vaccinated BALB/c mice. rHER2/neu-positive TUBO and 3T3/NKB cell lines and rHER2/neu-negative NIH/3T3 cells were stained with pooled sera collected at different time points from BALB/c mice immunized i.m. with 10 or 1 µg of rHER2/neu conjugated to NPX or emulsified with alum, or i.v. with 10 or 1 µg of the bioconjugate or the protein alone (6 mice per group). One year time point is not available for 10 µg i.v. immunization. Numbers in the upper right corners indicate MFI of sera staining.

We therefore were able to calculate antigen-specific IgG concentration by flow cytometry, based on a standard curve created by incubating 3T3/NKB with scalar concentrations of the Ab4 mAb (**Fig. 8**). Results are reported in **Table 4**. Again, these data demonstrate the superiority of NPX adjuvanticity over alum, since not only NPX-based vaccination strategies produced higher IgG titers, but also strong humoral responses were detectable with lower amount of antigen, as 1 µg of bioconjugate administered i.m. was sufficient to generate IgG titers comparable to those obtained with 10 µg of rHER2/neu emulsified with alum.

We could not perform a similar quantification with TUBO cells, since rHER2/neu expression on this cell line is not stable over time; moreover, we were not able to discriminate TUBO staining at

low concentrations of Ab4 mAb, as from the dilution of 0.050 µg/ml of Ab4 overlapping staining curves were detected (data not shown).



Sample	Dilution	Concentration (µg/ml)	MFI
	1:20000	0.005	6.24
	1:10000	0.010	13.7
	1:7000	0.014	18.3
	1:5000	0.020	29.9
	1:4000	0.025	38.0
	1:2000	0.050	125
	1:1500	0.070	196
	1:1000	0.100	279
	1:800	0.125	307
	1:600	0.167	1396

Figure 8. Example of standard curve generated for rHER2/neu-specific IgG titration. 3T3/NKB cells were incubated with scalar concentrations of the anti-rHER2/neu commercial Ab4 mAb, and the correlation between MFI and Ab4 concentration was analyzed as detailed in Materials and Methods section.

Dosage and route of administration	Sample	IgG concentration [µg/ml] of sera at the indicated time points (days)					
		0	14	30	90	150	360
10 µg i.m.	rHER2/neu+alum	0.817	0.955	5.536	4.221	2.645	1.271
	rHER2/neu-NPX	0.795	1.059	8.298	5.930	5.043	3.188
1 µg i.m.	rHER2/neu+alum	0.813	0.940	2.655	2.527	1.511	1.023
	rHER2/neu-NPX	0.808	0.995	5.306	2.465	1.758	1.353
10 µg i.v.	rHER2/neu	0.632	0.673	0.772	0.772	0.723	/
	rHER2/neu-NPX	0.616	1.233	1.777	1.582	1.480	/
1 µg i.v.	rHER2/neu	0.611	0.656	0.660	0.678	0.614	0.675
	rHER2/neu-NPX	0.607	0.957	2.719	1.592	1.471	1.283

Table 4. Quantification of rHER2/neu-specific IgG concentration by flow cytometry. Sera were collected from BALB/c mice that had been previously immunized i.m. with 10 or 1 µg of rHER2/neu conjugated to NPX or emulsified with alum, or i.v. with 10 or 1 µg of the bioconjugate or the protein alone. Serum IgG quantification was carried out as detailed in Materials and Methods section.

In contrast to what observed in ELISA test, vaccination of mice with PADRE-A3 conjugated to NPX or emulsified with alum induced humoral responses with a reduced capacity to bind the receptor

in its native conformation (**Fig. 9**). This result suggests that A3 peptide represents a good candidate for anti-rHER2/neu vaccination, but probably both A3 sequence and the vaccination protocol should be improved.

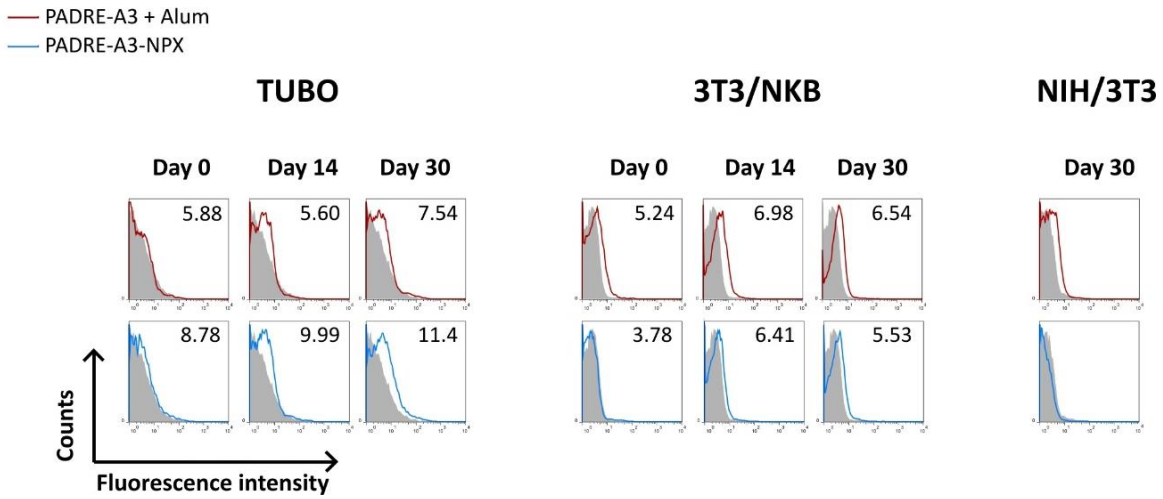


Figure 9. Flow cytometry analysis of rHER2/neu-positive or negative cell lines, stained with pooled sera from BALB/c mice immunized i.m. with NPX-conjugated PADRE-A3 peptide. rHER2/neu-positive TUBO and 3T3/NKB cell lines, and rHER2/neu-negative NIH/3T3 cells were stained with pooled sera collected from BALB/c mice immunized i.m. with 30 µg of PADRE-A3 peptide conjugated to NPX or emulsified with alum and analyzed by flow cytometry. Numbers in the upper right corners indicate MFI of sera staining.

Flow cytometry analysis confirmed the ability of alum and NPX-vaccination strategies to break tolerance against rHER2/neu in BALB-neuT mice (**Fig. 10**). Interestingly, only sera from NPX-vaccinated mice showed affinity for BALB-neuT-derived TUBO cell line. Moreover, as quantified by flow cytometry (**Table 5**), at day 30 NPX-vaccinated group displayed IgG titers that were 2-fold higher than those of the alum-vaccinated counterpart.

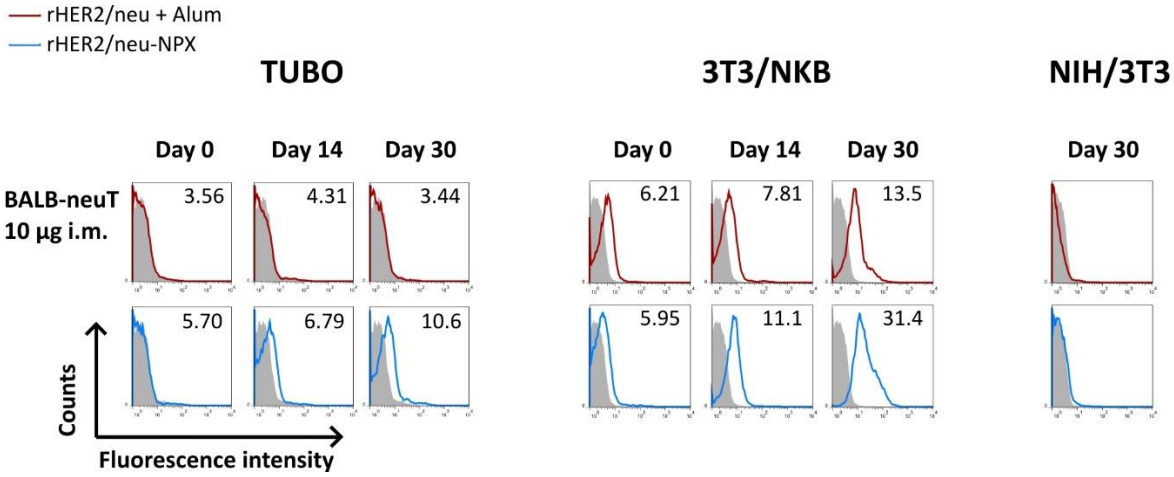


Figure 10. Flow cytometry analysis of TUBO, 3T3/NKB, and NIH/3T3 cells stained with pooled sera from vaccinated BALB-neuT mice. TUBO, 3T3/NKB, and NIH/3T3 cells were stained with pooled sera collected from BALB-neuT mice immunized i.m. with 10 µg of rHER2/neu conjugated to NPX or emulsified with alum as control. Numbers in the upper right corners indicate MFI of sera staining.

Dosage and route of administration	Sample	IgG concentration [µg/ml] at the indicated time points (days)		
		0	14	30
10 µg i.m.	rHER2/neu+alum	0.302	0.365	0.591
	rHER2/neu-NPX	0.291	0.495	1.300

Table 5. Concentration of pooled sera IgG antibodies as quantified by flow cytometry. Sera were collected from BALB-neuT mice that had been previously immunized i.m. with 10 of rHER2/neu conjugated to NPX or emulsified with alum. Serum IgG quantification was carried out as detailed in Materials and Methods section.

4. Complement-Dependent Cytotoxicity

Since tumor protection in both BALB/c and BALB-neuT mice is partly mediated through CDC (Rovero, Amici *et al.*, 2000, Hartman, Wei *et al.*, 2011), we investigated the ability of vaccine-induced antibodies to specifically mediate complement-dependent lysis of 3T3/NKB cell line.

NPX- and alum-induced immunoglobulines from both BALB/c and BALB-neuT mice showed a great ability to trigger CDC-mediated 3T3/NKB lysis (**Fig. 11**), whereas viability of rHER2/neu-negative NIH/3T3 cells was not affected (data not shown), thus demonstrating the specific recognition of the receptor. Interestingly, Abs from NPX-immunized mice led to levels of 3T3/NKB

lysis that were two to three times greater than those observed with sera from alum-vaccinated groups, suggesting that rHER2/neu-NPX induced more efficient effector immune responses. The difference in CDC functionality are likely due to the difference in IgG subtypes content of mouse sera, and in particular in IgG2a and IgG2b subclasses that are considered the most potent inducer of CDC in mice (Wittman, Woodburn *et al*., 2006; Chabner and Longo, 2011).

Despite the low concentration detected for total IgG content, sera from vaccinated BALB/c mice displayed an improved capacity to trigger CDC activity at 1 year after priming, in particular for NPX groups. Indeed, at this time point sera from mice vaccinated i.m. with 10 µg of bioconjugate mediated up to 90% of specific cell lysis, suggesting that NPX-vaccination strategies can efficiently stimulate and "mature" antibody-mediated effector functions.

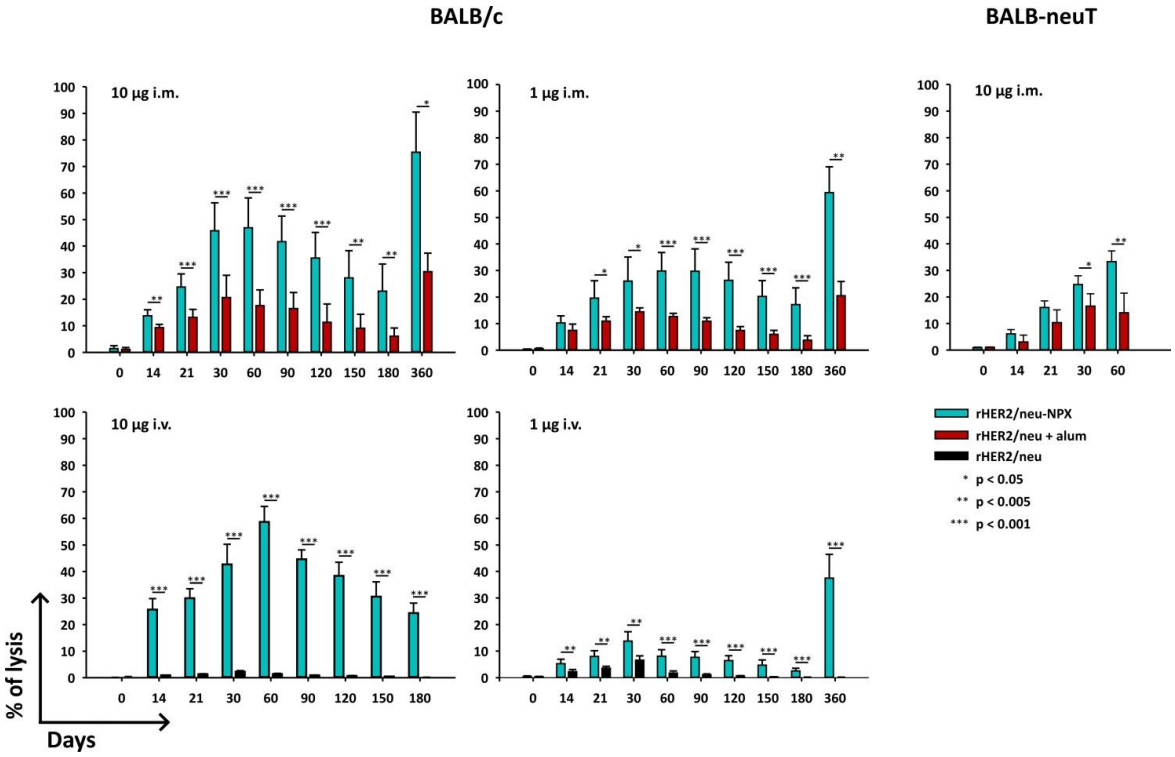


Figure 11. CDC-mediated 3T3/NKB lysis by sera from BALB/c and BALB-neuT vaccinated mice. Sera were collected at different time points from BALB/c and BALB-neuT vaccinated mice (6 and 4 mice per group, respectively). rHER2/neu-positive, ^{51}Cr-labeled 3T3/NKB cells were incubated with 1:100 dilutions of individual sera collected at different time points from rHER2/neu-immunized mice. Then, rabbit complement was added and cell lysis was evaluated in a ^{51}Cr release assay, as detailed in Materials and Methods section. * P<0.05; ** P<0.005; *** P<0.001, Student's *t* test for independent samples.

5. Cytokine production

IgG subclasses content suggested that i.m. administration of rHER2/neu-NPX induced both Th1 and Th2 immune responses, whereas alum resulted in a Th2-skewed immune response. To assess this hypothesis, we evaluated the cytokine production by splenocytes from BALB/c mice vaccinated i.m. with 10 μg of rHER2/neu conjugated to NPX or emulsified with alum, according to our standard vaccination protocol. Control group was represented by non-vaccinated BALB/c mice. Splenocytes were harvested at day 30 and stimulated *in vitro* with rHER2/neu protein, rHER2/neu-expressing TUBO and 3T3/NKB cells as positive controls, or with NIH/3T3 cells or medium alone as negative controls. Supernatants were harvested after 24 and 72 h, and their cytokine content (IFN-γ, IL-1β, IL-2, IL-6, IL-10, IL-12p70, IL-17, and TNFα) was analyzed by ELISA test.

Splenocytes from vaccinated mice incubated with NIH/3T3 cells or medium alone, and spleen cells from non-vaccinated mice produced negligible levels of cytokines (data not shown), thus demonstrating that cytokine production was exquisitely rHER2/neu-specific. Of note, splenocytes of mice immunized with alum produced typical Th2-type cytokines, namely IL-6 and IL-10 (**Fig. 12**). In addition, high levels of TNFα, INF-γ and IL-1β were detected, suggesting the probable activation of the NLRP3 inflammasome by alum, which triggered the production of these inflammatory cytokines (**Fig. 13**). Spleen cells from NPX-vaccinated mice disclosed high levels of all tested cytokines. In particular, NPX elicited stronger titers of the Th1-type cytokines IL-12p70, IFN-γ and IL-2, thus suggesting that NPX might also stimulate the differentiation and expansion of CTL effectors. Interestingly, no statistical difference was observed in the production of IL-6 and IL-10 between the two adjuvants, confirming that NPX stimulated also a strong Th2-type immune response. Indeed, stimulations of TLRs activates common pathways characterized by the production of both Th1- and Th2-type cytokines (**Fig. 13**). The un/balance between the two responses relies on the type and number of TLRs that are engaged, since the simultaneous stimulation of two or more TLRs can give rise to synergistic, antagonistic, or additive effects (Mäkelä, Strengell *et al*., 2009). NPX exerts its adjuvanticity by activating two types of TLRs: as a result, strong, synergistic and very balanced boost for both Th1 and Th2 immune responses is elicited.

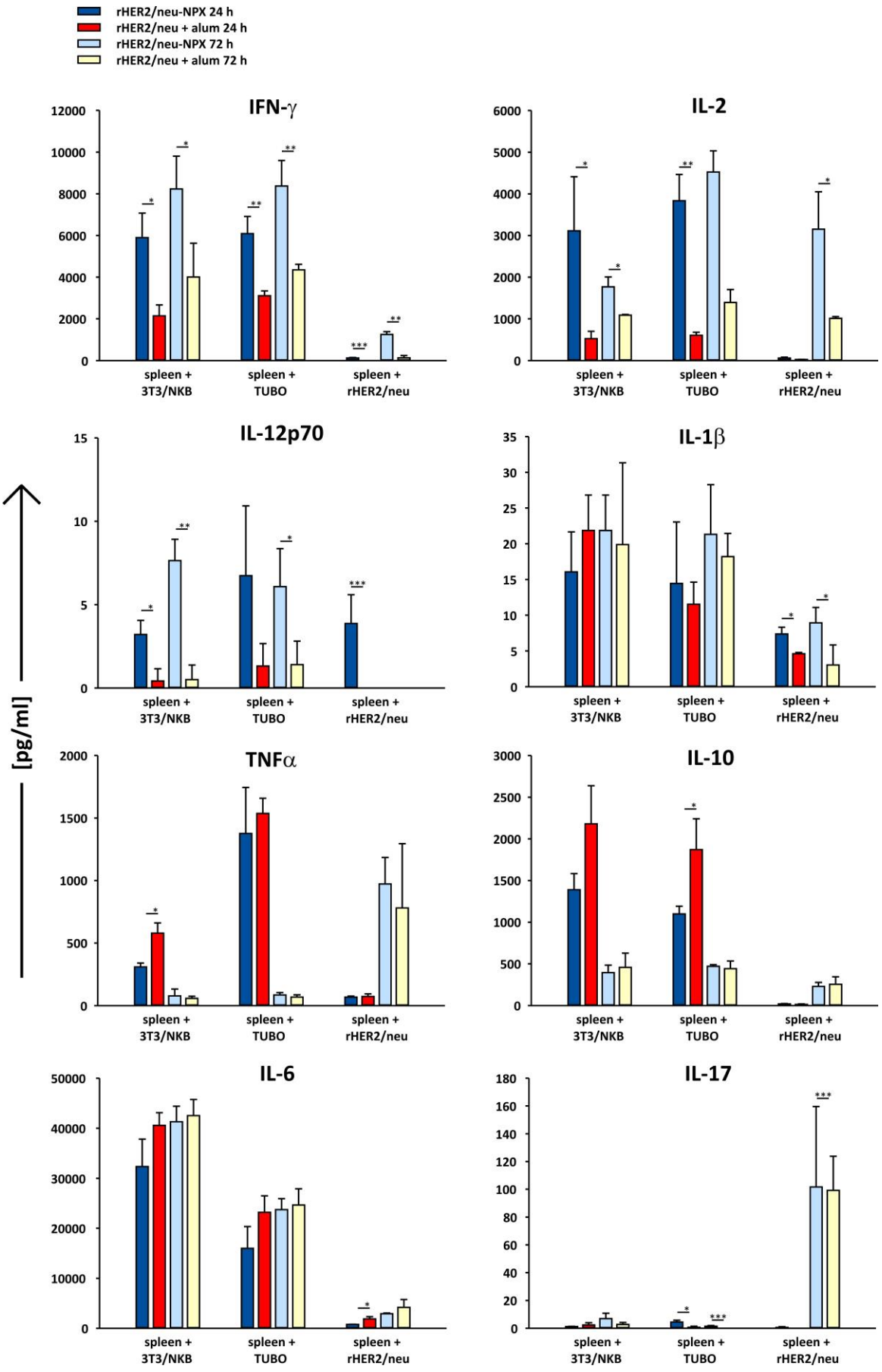

rHER2/neu-NPX 24 h
rHER2/neu + alum 24 h
rHER2/neu-NPX 72 h
rHER2/neu + alum 72 h
IFN-γ
IL-2
IL-12p70
IL-1β
TNFα
IL-10
IL-6
IL-17
[pg/ml]
spleen + 3T3/NKB
spleen + TUBO
spleen + rHER2/neu

Figure 12. Cytokine production by spleen cells 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 rHER2/neu-NPX or rHER2/neu emulsified in alum (3 mice per group). Splenocytes were restimulated for 24 or 72 h with 1 µg/ml of rHER2/neu protein, with TUBO or 3T3/NKB cell lines. The ratio of splenocytes to APC was 10:1. Cytokine content was assessed by ELISA test. Where concentration values are not reported, cytokine content was below the detection threshold level for this method. * P<0.05; ** P<0.005; *** P<0.001, Student's *t* test for independent samples.

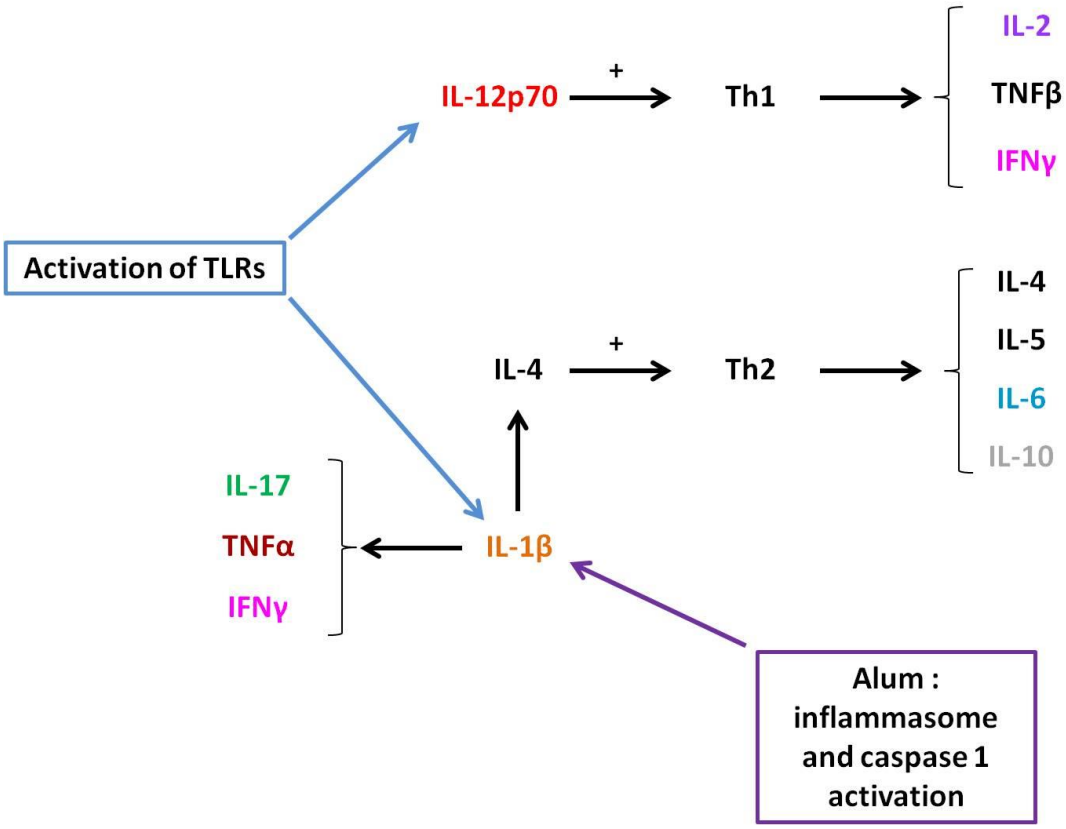


Figure 13. Cytokine production induced by alum and TLR ligands. Alum adjuvanticity seems to principally reside in the activation of the NLRP3 inflammasome and consequently of caspase 1, which in turn activates precursors of the IL-1 family, including the proinflammatory cytokine IL-1β. (McKnee, Munks *et al*., 2009). IL-1β supports the proliferation and expansion of Th cell precursors, and induce T cell production of IFN-γ, TNFα and IL-17, but also IL-4, which is involved in the differentiation of naïve T cells into Th2 cells. Th2 responses are characterized by the production of IL-4, IL-5, IL-6, and IL-10 (Hebel, Rudolph *et al*., 2011). Stimulation of TLRs activates a common signaling pathway that culminates in the induction of inflammatory cytokines such as TNFα, IL-6, IL-1β, and IL-12p70. This latter cytokine drives the differentiation of naïve T cells into Th1 cells, characterized by the production of IL-2 and IFN-γ, which in turn stimulate the differentiation and expansion of CTL effectors. The balance between Th1 and Th2 responses depends on the type and number of TLRs activated (Kawai and Akira, 2006; Mäkelä, Strengell *et al*., 2009).

6. Prophylactic and therapeutic models of vaccination: TUBO challenge in BALB/c mice

To investigate whether vaccination with rHER2/neu-NPX induces antitumoral immunity and protects animals from tumor growth, BALB/c mice were vaccinated i.m. at day 0, 14, and 21 with 10 or 1 µg of rHER2/neu-NPX or rHER2/neu emulsified in alum, and challenged at day 30 with $1x10^5$ TUBO cells. Control group consisted of mice that received only TUBO injection. NPX vaccination strategies effectively prevented tumor growth, since all animals vaccinated with 10 µg of the bioconjugate remained tumor free (**Fig. 14 A**), while only two mice immunized with the lower dose of rHER2/neu-NPX developed tumors (data not shown). Nonetheless, vaccination significantly increased their survival when compared to control and alum-vaccinated groups (**Fig. 14 C**). By contrast, alum failed to confer protection against TUBO cells. Indeed, all animals in these groups developed continuously growing tumors, even though with a delayed tumor growth kinetics when compared to controls. However, this preventive effect did not significantly increase survival since all alum-vaccinated and control mice had to be sacrificed within 5 weeks (**Fig. 14 A and C**).

To determine whether the protective immune response elicited by rHER2/neu-NPX vaccination resulted in memory induction and hence long-term protection, mice that survived from tumor challenge were rechallenged with $1x10^6$ TUBO cells 3 months after the first challenge. Control group was represented by mice that received only TUBO injection. Impressively, rHER2/neu-NPX vaccination succeeded in conferring long-term protection. Of note, the majority (4/6) of mice vaccinated with 10 µg of the bioconjugate rejected the tumors, while both dosages significantly delayed tumor onset and growth (**Fig. 14 B and D**).

These data strongly supports the concept that NPX-adjuvanted vaccination is able to induce strong immune responses and long-term immune memory capable of protecting the host from tumor relapse.

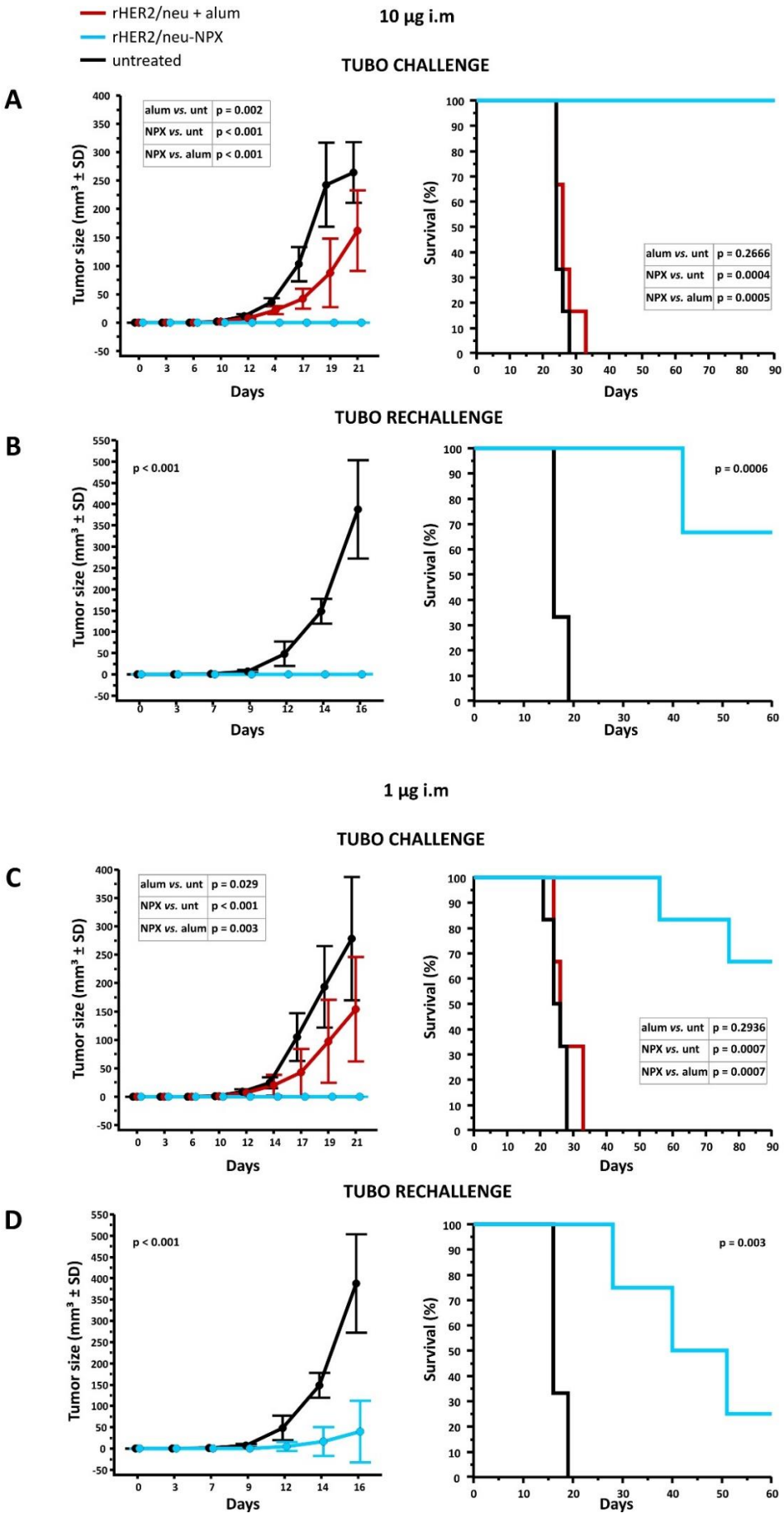

rHER2/neu + alum
rHER2/neu-NPX
untreated
10 µg i.m
TUBO CHALLENGE
A
alum vs. unt p = 0.002
NPX vs. unt p < 0.001
NPX vs. alum p < 0.001
Tumor size (mm³ ± SD)
Days
Survival (%)
alum vs. unt p = 0.2666
NPX vs. unt p = 0.0004
NPX vs. alum p = 0.0005
TUBO RECHALLENGE
B
p < 0.001
p = 0.0006
1 µg i.m
TUBO CHALLENGE
C
alum vs. unt p = 0.029
NPX vs. unt p < 0.001
NPX vs. alum p = 0.003
alum vs. unt p = 0.2936
NPX vs. unt p = 0.0007
NPX vs. alum p = 0.0007
TUBO RECHALLENGE
D
p < 0.001
p = 0.003

Figure 14. Prophylactic model of vaccination. (A and C) BALB/c mice (6 animals per group) were vaccinated i.m. at day 0, 14, and 21 with 10 or 1 μg of rHER2/neu-NPX or rHER2/neu emulsified in alum, and challenged at day 30 with $1x10^5$ TUBO cells. **(B and D)** Mice that survived from tumor challenge were evaluated for long-term protection by rechallenge with $1x10^6$ TUBO cells 3 months after the first tumor cell inoculation. In both experiments, control groups were represented by untreated mice that receive only TUBO injection. Tumor growth was monitored three times per week, and when tumor volumes were >400 mm^3 mice were sacrificed. Kinetics of tumor growth (left panels) and survival of mice (right panels) are represented.

To assess if NPX vaccination is also effective in the therapeutic setting, BALB/c mice were first challenged with $1x10^5$ TUBO cells, and when they all had an established tumor of ~3 mm in diameter (day 12), were vaccinated i.m. at day 12, 19, and 26 with 10 or 1 μg of rHER2/neu conjugated with NPX or emulsified with alum. Control group was represented by mice that were only challenged with tumor. Both NPX- and alum-adjuvanted vaccinations elicited therapeutic antitumor effects, since tumor growth in vaccinated mice was substantially reduced as compared to control group; nevertheless, these effects were more pronounced and were statistically significant only for NPX-vaccinated mice. Interestingly, 1 μg of the bioconjugate was sufficient to elicit antitumor immune responses able to prolong survival of treated mice (**Fig. 15**).

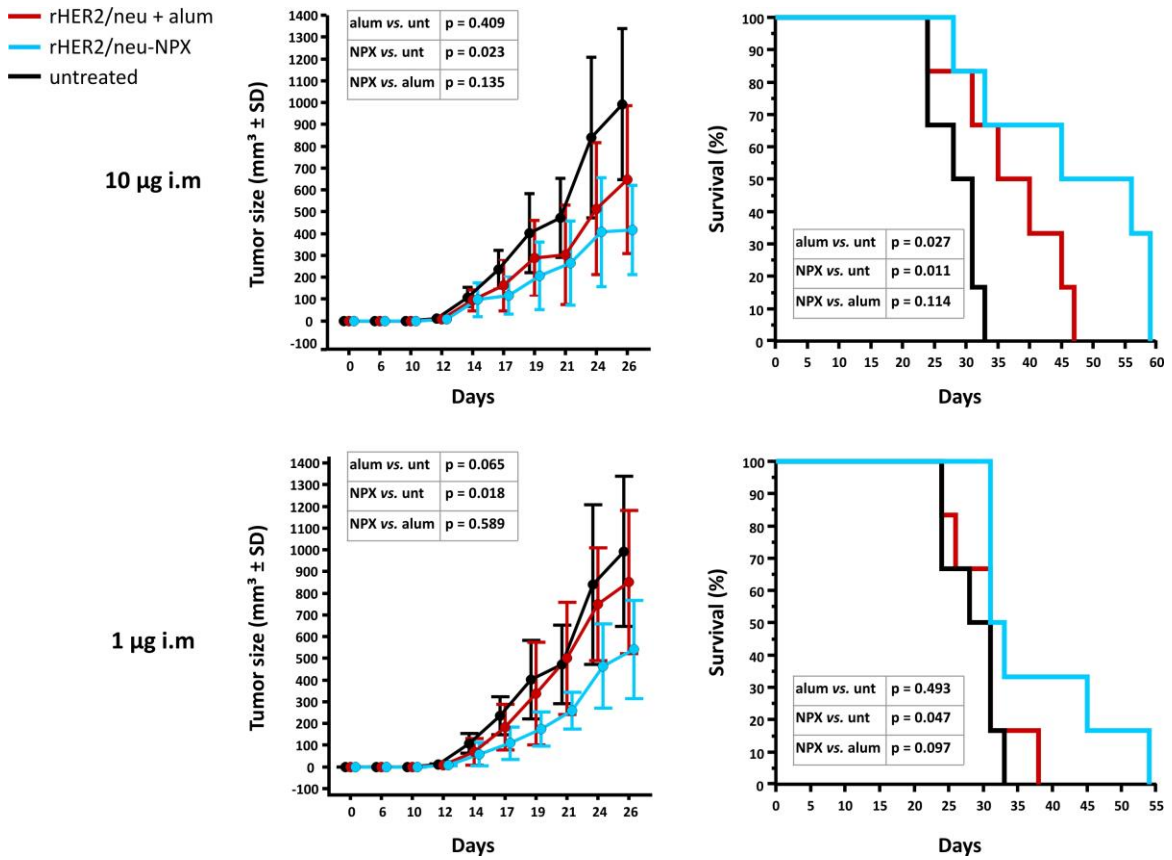


Figure 15. Therapeutic model of vaccination. BALB/c mice (6 animals per group) were challenged with 1x10^5 TUBO cells, and when all tumors were ~3 mm in the largest diameter mice were vaccinated i.m. at day 12, 19, and 26 with 10 or 1 µg of rHER2/neu-NPX or rHER2/neu emulsified in alum. Control group was represented by untreated mice that receive only TUBO injection. Tumor growth was monitored three times per week, and when tumor volumes were >1000 mm^3 mice were sacrificed. Kinetics of tumor growth (left panels) and survival of mice (right panels) are reported.

7. Evaluation of CTL responses

Recent studies indicate that the induction of CTL responses, which are required for efficient antitumor activity, can be finely stimulated by TLR ligands. Thus, we explored the NPX ability to induce antigen-specific CTL responses and to assess if this cell population contributes to tumor protection. To this end, we isolated splenocytes of BALB/c mice immunized i.m. with 10 or 1 µg of rHER2/neu emulsified in alum or conjugated to NPX, and also from all animals (vaccinated and control groups) that were monitored in the previously described experiments of prophylactic model of TUBO challenge and rechallenge. Following *in vitro* restimulation with TUBO, 3T3/NKB, and NIH/3T3 cells, the potentiality of spleen lymphocytes to specifically kill rHER2/neu-expressing cells was determined by using a ^{51}Cr release assay. The results reported in **Fig. 16** are represented as percentage of specific lysis at different effector:target (E:T) ratios.

No CTL response was observed in mice that were only immunized with the two adjuvants, in spleens stimulated with NIH/3T3, and in both alum-vaccinated and control challenged groups (data not shown). By contrast, a strong CTL activity was detected in NPX-vaccinated animals that developed tumors during TUBO challenge experiments (**Fig. 16, orange lines**) or that survived from both tumor inoculations (**Fig. 16, blue lines**). Cell lysis was high for both rHER2/neu-NPX dosages, and the specificity of the response was verified by the inhability of splenocytes to lyse the rHER2/neu-negative NIH/3T3 cell line (data not shown). Interestingly, tumor-bearing mice of TUBO rechallenge experiments did not developed any detectable CTL response (**Fig. 16, black lines**).

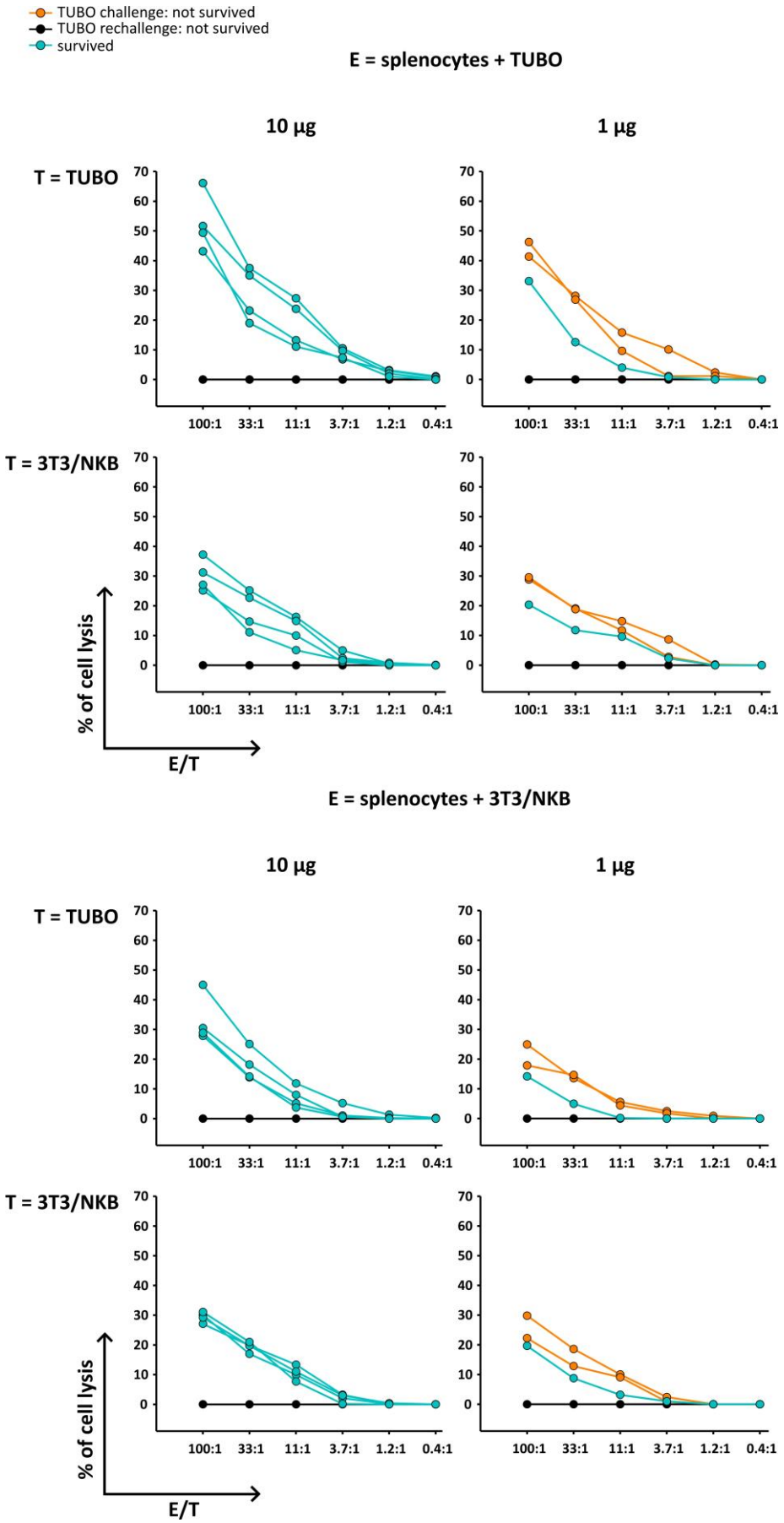

TUBO challenge: not survived
TUBO rechallenge: not survived
survived
E = splenocytes + TUBO
10 µg
1 µg
T = TUBO
T = 3T3/NKB
% of cell lysis
E/T
100:1
33:1
11:1
3.7:1
1.2:1
0.4:1
E = splenocytes + 3T3/NKB
10 µg
1 µg
T = TUBO
T = 3T3/NKB
% of cell lysis
E/T

Figure 16. Cytotoxic T cell responses from NPX-vaccinated and TUBO challenged BALB/c mice. BALB/c mice (6 per group) were vaccinated i.m. at day 0, 14, and 21 with 10 or 1 μg of rHER2/neu-NPX. At day 30 they were challenged with $1x10^5$ TUBO cells. Mice that survived were rechallenged with $1x10^6$ TUBO cells 3 months after the first tumor cell inoculation. Spleen cells of mice that did not survived to TUBO challenge (orange lines), TUBO rechallenge (black lines), or that were tumor-free at the end of the experiments (blue lines) were restimulated *in vitro* with TUBO or 3T3/NKB cells, and evaluated for their rHER2/neu-specific lytic activity against the same cell lines by ^{51}Cr release assay. T: target cells; E: effector cells.

The discrepancy between CTL responses observed in only vaccinated and tumor challenged groups suggests that NPX-based vaccination may promote the differentiation of a quite limited population of $CD8^+$ effectors, which expanded after the strong boost conferred by challenge with rHER2/neu-positive TUBO cells. CTL activity seems to contribute to tumor protection or tumor growth delay, as the only groups that survived from tumor inoculation or that developed tumors with a slow growth rate were those vaccinated with NPX (**Fig. 14**). However, additional experiments are required to better understand the role of CTL activity in these responses, since probably tumor protection is conferred by a fine cross-talk between humoral and cellular responses. Indeed, CTL activity alone may not be sufficient to induce protection, since tumor growth was observed despite the presence of detectable CTL responses for some NPX-vaccinated animals (**Fig. 16, orange lines**). On the other hand, $CD8^+$ T cell activity may significantly contribute to tumor prevention, since the long-term protection observed in mice that survived from tumor rechallenge seemed at least in part due to the induction of memory CTL responses (**Fig. 16, blue lines**). The absence of detectable CTL activity observed in mice that survived to tumor challenge but not to tumor rechallenge is controversial (**Fig. 16, black lines**). A plausible explanation is that these mice generated CTL responses able to protect them from the first challenge, but then they failed to establish CTL memory. These mice may also have developed immune-suppressive mechanisms that inhibited CTL activity during tumor rechallenge. On the other hand, antigen-specific T cells can be undergone activation-induced cell death (AICD) due to the overwhelming antigen load encountered during rechallenge.

8. Prevention of spontaneous tumor growth: BALB-neuT monitoring

Although mouse models based on tumor cell grafts are useful to assess the efficacy and functionality of cancer vaccines, such models do not fully recapitulate the situation of human cancers, which arise in an immunological environment characterized by central and peripheral tolerance toward the tumor antigen. Hence, we investigated the ability of NPX-based vaccination to confer tumor protection also in BALB-neuT mice, which represent an immunotolerant model of

spontaneous and aggressive rHER2/neu-positive cancer. Thus, 8 week-old female BALB-neuT mice were immunized i.m. with 10 µg of rHER2/neu-NPX or rHER2/neu emulsified with alum according to our standard protocol, and appearance of spontaneous tumors was monitored three times per week. Control group was represented by non-vaccinated BALB-neuT mice.

By week 15 of age all control mice exhibited the first measurable tumors, and by week 25 invasive tumors in all 10 mammary glands were detectable (**Fig. 17, left panel**). Alum completely failed to confer any tumor protection, since both tumor multiplicity and tumor-free survival curves overlapped those of the control group. By contrast, NPX vaccination resulted in a significant increase in tumor-free survival (**Fig. 17, right panel**), in a marked delay (about 3 weeks) in the appearance of macroscopically detectable tumors in the mammary glands, and in a much lower number of mammary glands involved as compared with alum-vaccinated and control groups (**Fig. 17, left panel**). Of note, all NPX-immunized animals had significantly smaller tumors compared to those of the counterparts (data not shown).

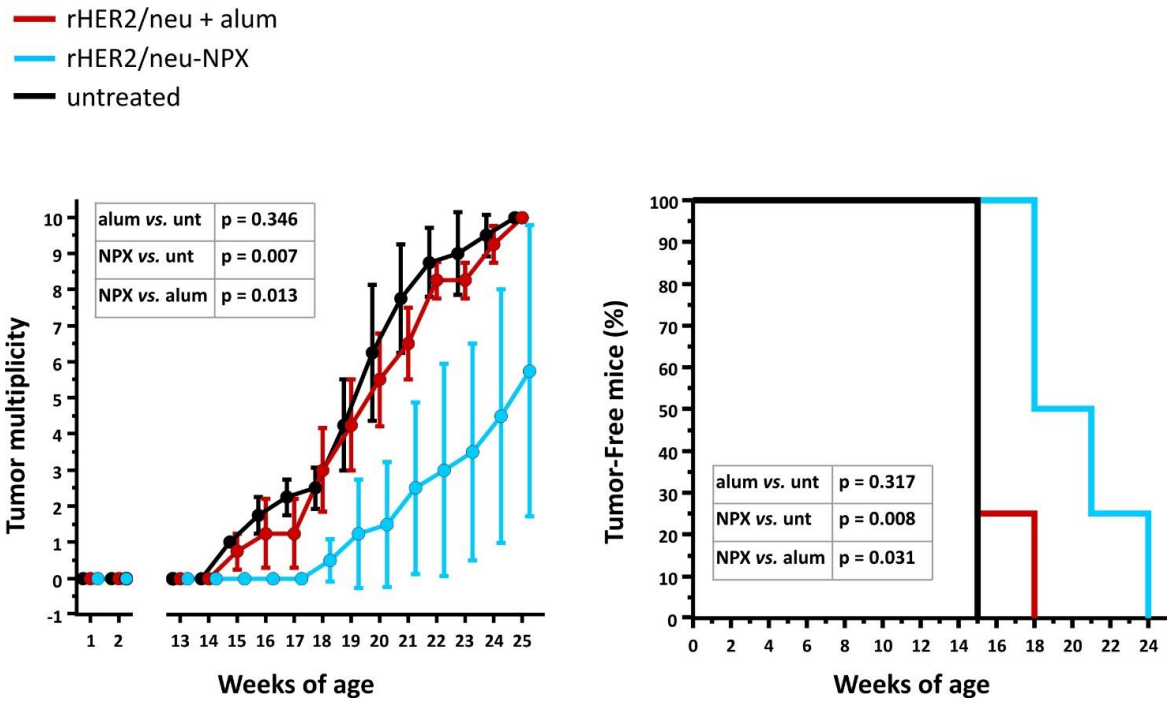


Figure 17. Prevention of spontaneous tumors by NPX-based vaccination. Eight week-old female BALB-neuT mice (4 animals per group) were vaccinated i.m. at week 8, 10, and 11 of age with 10 µg of rHER2/neu-NPX or rHER2/neu emulsified in alum. Control group was represented by untreated mice. All animals were monitored for tumor appearance by manual examination of the mammary glands 3 times per week. Measurable masses >2 mm in diameter were regarded as tumors. Data are reported as tumor multiplicity (cumulative number of tumors per number of mice in each group) per week of age (left panel), and as percentage of tumor-free mice (right panel).

Discussion

The use of proteins or peptides as immunogens is attractive for the development of vaccines, and in particular for cancer vaccines, but requires efficient and safe adjuvant formulations to overcome their weak immunogenicity (Miconnet, Coste *et al*., 2001). Indeed, antibodies elicited in animals have generally shown low affinity for the native protein (Dakappagari, Douglas *et al*., 2000), and the stimulated cellular and humoral responses seldom protected animals against tumor challenge (Buhrman, Jordan *et al*., 2013). Thus, adjuvants for cancer vaccines need to be more potent than for prophylactic vaccines, but most of them have resulted excessively toxic for clinical use (Mesa and Fernández, 2004). Moreover, the majority of commercially available adjuvants proved to be ineffective in inducing successful antitumor effects, since they preferentially stimulate Th2-skewed immune responses, without promoting CTL activity (Nava-Parada, Forni *et al*., 2007). Indeed, recent studies indicate that efficient antitumor effects require balanced Th1 and Th2 immune responses, characterized by the presence of a strong CTL activity, and the production of IgG subclasses with specific effector functions (Ellyard, Simson *et al*., 2007; Mocellin, Marincola *et al*., 2004). For example, tumor prevention in mice is associated with high levels of IgG2a and IgG2b subclasses, which are considered the most potent inducers of CDC and ADCC in rodents (Wittman, Woodburn *et al*., 2006; Chabner and Longo, 2011).

TLR ligands are emerging as a new class of vaccine adjuvants due to their ability to orchestrate the cross-talk between innate and adaptive immunity. In particular, TLR agonists induce the maturation of DCs and finely regulate the Th1/Th2 balance, thus promoting potent and long-lasting humoral and cellular responses (Ito and Chang, 2013). Among TLR agonists, NPs and their derivatives originating from plants, animals, and microbes have been studied for many years as they offer a unique set of advantages over conventional adjuvants. In fact, by acting as DAMPs or PAMPs, they induce specific antitumor immune responses due to their ability to interact with TLR, and are also biocompatible, biodegradable, non-toxic, non-immunogenic, and non-inflammatory (Adams and Mallapragada, 2014).

In this work, we showed that the natural polymer we developed (NPX) can be regarded as a good adjuvant candidate for the design of safe and efficient anticancer vaccine formulations, as demonstrated by results obtained against the rat form of HER2/neu oncoantigen. In fact, NPX displayed a very good safety profile as no local side effects occurred in vaccinated mice; in particular, unlike alum, it did not triggered any visible uncontrolled inflammatory reaction at the site of injection, thus preserving muscle integrity.

Both alum and NPX-adjuvanted formulations fostered high humoral immune responses against rHER2/neu, and the robustness of the antibody response induced in vaccinated groups was evaluated in terms of isotype switching, a process that involves Th cell engagement. In this regard, both adjuvants induced high production of different IgG subclasses; however, despite similar IgG1 levels in both groups, NPX-vaccinated mice disclosed higher IgG2a and IgG2b titers for both the i.m. and i.v. administration routes. The superiority of NPX as an adjuvant was also demonstrated in mice vaccinated with the rHER2/neu-derived A3 peptide. Indeed, while this bioconjugate elicited a strong boost for IgG production, alum completely failed in enhancing peptide immunogenicity.

The strong elicitation of the three IgG subclasses observed in NPX-vaccinated mice suggested that NPX likely promotes both Th1 and Th2 responses in mice. In fact, the IgG2a/IgG1 ratio clearly demonstrated that alum induced a typical Th2-skewed immune response, while mice immunized with the bioconjugate displayed more balanced Th1 and Th2 responses. Cytokine production confirmed these data, as NPX elicited higher titers of the Th1-type cytokines IL-12p70, IFN-γ and IL-2, indicating that this adjuvant may also stimulate the differentiation and expansion of CTL effectors. Moreover, both adjuvants boosted the production of the Th2-related IL-6 and IL-10 cytokines.

The different quality and quantity of IgG subclasses and their superior ability to recognize rHER2/neu in its native conformation, likely reflected on the better capability of NPX-induced antibodies in triggering complement-mediated specific lysis of rHER2/neu-positive cells. In fact, NPX very effectively induced humoral responses that persisted over time, and also selected mature B cell clones secreting antibodies that mediate improved effector functions.

NPX-adjuvanted vaccination showed to be effective in both the prophylactic and therapeutic settings; indeed, the immune responses elicited prevented or significantly delayed tumor growth. Of note, in prophylactic vaccination 10 μg of rHER2/neu-NPX were sufficient to elicit a protective long-term immune response in mice, as shown by tumor rechallenge experiments. Interestingly, antitumor responses appeared in part to be mediated by NPX ability to induce cell-mediated responses, since alum-vaccinated groups, which did not display any detectable CTL activity, completely failed to protect mice from tumor challenge. Nevertheless, future experiments with $CD8^+$ or $CD4^+$ T cell-depleted or knock-out mice will be instrumental to better elucidate the role played by these populations in the antitumor responses observed.

Finally, both alum- and NPX-adjuvanted vaccines proved to be effective in breaking immune tolerance against rHER2/neu in transgenic mice, with NPX-vaccinated mice displaying IgG titers

that were at least two-fold higher than those observed with alum. All of the three IgG subclasses were produced by vaccinated groups; interestingly, NPX vaccination resulted in more balanced Th1 and Th2 responses in transgenic mice than in BALB/c animals. Of note, only NPX-induced antibodies were able to bind rHER2/neu in its native conformation. This probably reflected on the ability of the bioconjugate to successfully protect mice from spontaneous tumor growth, and to significantly prolong tumor-free survival. By contrast, alum-based vaccination failed to confer tumor protection in BALB-neuT mice.

Taken together, our data show that NPX is a safe and powerful adjuvant that could be exploited for the development of new HER2/neu vaccination strategies. In fact, NPX efficiently enhanced the magnitude, breadth, quality, and longevity of specific humoral and cellular immune responses to antigens, without causing toxicity. Importantly, these effects can be achieved even with a strongly reduced antigen dose.

Experiments are currently underway to compare the immunogenicity of NPX with that of other clinical-grade adjuvants, such as MF59, and to better assess its mechanism of action. Moreover, alternative vaccination protocols are under development in order to further improve tumor protection in BALB-neuT mice.

Abbreviations

A

Abs: antibodies

ACT: adoptive T cell therapy

ADCC: antibody-dependent cell cytotoxicity

AFP: alpha-fetoprotein

AICD: activation-induced cell death

AIM2: absence in melanoma 2-like receptor

Akt: protein kinase B

AML: acute myeloid leukaemia

APC: antigen-presenting cells

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

B

BAGE: B antigen family

BCR: B-cell receptor

C

CARs: chimeric antigens receptors

CARD: caspase activation and recruitment domain

CCR7: C-C chemokine receptor type 7

CDC: complement-dependent cytotoxicity

CEA: carcinoembryonic antigen

CLL: chronic lymphocytic leukemia

CLRs: C-type lectin receptors

CpG: cytosine phosphoguanosine

CR: cysteine-rich

CRPC: castration-resistant prostate cancer

Abbreviations

CTL: cytotoxic $CD8^+$ T lymphocytes

CTLA-4: cytotoxic T-lymphocyte antigen-4

CXCL13: C-X-C motif chemokine 13

CXCR5: C-X-C chemokine receptor type 5

D

DAMPs: damage-associated molecular patterns

DCs: dendritic cells

DCregs: regulatory dendritic cells

dsRNA: double-stranded RNA

DT: diphtheria-tetanus

E

EBV: Epstein-Barr virus

EBNA: EBV-encoded nuclear antigen

ECD: extracellular domain

ECM: extracellular matrix

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

F

FasL: Fas ligand

FBS: fetal bovine serum

FDA: Food and Drug Administration

FDCs: follicular dendritic cells

fHA: hyaluronan fragments

FISH: fluorescence in situ hybridization

G

GAGE: G antigen family

GCs: germinal centers

GM-CSF: granulocyte-macrophage colony-stimulating factor

H

HA: haemagglutinin

HB: heparin-binding

HBsAg: recombinant HBV surface antigen

HBV: hepatitis B virus

HER2: human epidermal growth factor receptor 2

hGH: human growth hormone

HLA: human leukocyte antigen

HMGB1: high motility group box 1

HPV: human papillomavirus

HTLV: human T-cell lymphotropic virus

I

ICD: intracellular domain

iDCs: immature dendritic cells

IDO: indoleamine 2,3-dioxygenase

IFA: incomplete Freund's adjuvant

IFN: interferon

Ig: immunoglobuline

IHC: immunohistochemistry

IL: interleukin

i.m.: intramuscular

ISCOMs: immunostimulatory complexes

ISS: immunostimulatory sequences

ITAM: immunoreceptor tyrosine-based activation motif

L

LD: ligand-domain

LPS: lipopolysaccharide

LRR: leucine-rich repeated

M

M2: macrophage type 2

mAbs: monoclonal antibodies

MAGE: melanoma antigen family

MAPK: mitogen-activated protein kinase

MDA5: melanoma differentiation associated gene 5

mDCs: mature dendritic cells

MDSCs: myeloid-derived suppressor cells

MHC: major histocompatibility complex

MMTV: mouse mammary tumor virus

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

N

NF-κB: nuclear factor-κB

NHL: non-Hodgkin lymphoma

NK: natural killer

NLRs: NOD-like receptors

NPs: natural polymers

O

ODN: oligodeoxynucleotides

OVA: ovalbumin

o/w: oil-in-water

P

PADRE: pan-DR Th epitope

PAMPs: pathogen-associated molecular patterns

PAP: prostate acid phosphatase

PBS: phosphate buffered saline

PD-1: programmed cell death 1

PD-L1: programmed death-ligand 1

pDC: plasmacytoid dendritic cells

PI3K: phosphatidylinositol 3-kinase

PLG: polylactide coglycolide

PMNs: polymorphonuclear cells

Poly(I:C): polyiosinic:polycytidylic acid

Poly-ICLC: poly-L-lysine and carboxymethylcellulose

PRRs: pattern recognition receptors

PTLD: post-transplant lymphoproliferative disease

R

RAG: recombination activating gene

RIG-I: retinoic-inducible gene-1

RLRs : RIG-I-like receptors

S

SCCHN: squamous cell cancer of the head and neck

SOD: superoxide dismutase

Span 85: sorbitan trioleate 85

SPF: Specific Pathogen Free

STAT: signal transducer and activator of transcription

T

TA: tibialis anterior

TAA: tumor-associated antigen

TAMs: tumor-associated macrophages

TCR: T-cell receptor

Tfh: $CD4^+$ T follicular helper lymphocytes

TGF: transforming growth factor

Th: $CD4^+$ T helper lymphocytes

TiDCs: tumor-associated immature dendritic cells

TILs: tumor infiltrating lymphocytes

TLRs: Toll-like receptors

TNF: tumor necrosis factor

TRAIL-R1: tumor necrosis factor-releated apoptosis-inucing ligand receptor 1

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

TSA: tumor-specific antigens

Tween 80: polysorbate 80

V

VEGF: vascular endothelial growth factor

W

w/o: water-in-oil

Z

ZAP-70: zeta-chain-associated protein kinase 70

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