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"Studies on the synergistic protective activity of cancer antigens associated to engineered bacterial Outer Membrane Vesicles"

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‘Declaration of authorship:

I, Luisa Ganfini, confirm that either I have partially contributed to, or I have been fully responsible for, this work *and the use of all material from other sources has been properly and fully acknowledged.’*

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ABSTRACT

Introduction

Bacterial Outer Membrane Vesicles (OMVs) are naturally produced by all Gram-negative bacteria and are emerging as an attractive vaccine platform. The exploitation of OMVs in cancer immunotherapy was tested by decorating them with cancer epitopes and by following their capacity to elicit protective immune responses, alone or in combination, using cancer mouse models. Furthermore, we tested whether cancer cell-derived exosomes (TEXs) and OMVs can form complexes and whether such complexes, which carry TEX-associated tumor antigens and OMV-associated immune-stimulatory molecules, elicit anti-tumor immune responses *in vivo*.

Results

Immunization with OMVs engineered with two B cell epitopes (EGFRvIII and D8-mFAT1) induced tumor growth inhibition after mouse challenge with cell lines expressing the corresponding epitopes. Furthermore, mice immunized with engineered OMVs carrying two cancer epitopes, the EGFRvIII B cell epitope and the M30 CD4+ T cell epitope, were completely protected from EGFRvIIIB16F10 cell line expressing both epitopes, indicating the importance of multi-antigen immunization in cancer immunotherapy. Furthermore TEXs-OMVs immunization induced antibody responses against exosome antigens with a Th1-type profile. Finally, the combination of CT26-derived TEXs and MBP-D8-mFAT1 OMVs elicited synergistic protective activity against mouse challenge with CT26 cell line.

Conclusions

OMVs decorated with tumor antigens elicit antigen-specific, protective anti-tumor responses in mice. The synergistic protective activity of multiple epitopes simultaneously administered with OMVs, either by direct OMV engineering or by TEX-OMV combination, demonstrates the attractiveness of the OMV platform in cancer immunotherapy.

1 INTRODUCTION

Cancer is the second leading cause of death worldwide, accounting for 8.8 million deaths in 2015 (World Health Organization (WHO) statistics[1]).

With the continuing growth and aging of the world's population, the global burden of new cancer cases is estimated to rise by about 70% over the next two decades[1,2], with more than 50% of the people above 75 years of age expected to die because of cancer. The American Cancer Society (ACS) estimates that roughly 1.7 million new cases of cancer will be diagnosed in the U.S. in 2017 and more than 15 million Americans living today have a cancer history[3]. The Agency for Healthcare research and Quality (AHRQ) estimates that the direct medical costs (total of all health care costs) for cancer in the U.S. in 2014 were 87.8 billion USD: 58% of this cost for hospital outpatient or doctor office visits and 27% of this cost for inpatient hospital stays. The cost of cancer care is a topic at the center of a national discourse on fiscal responsibility and resource allocation. According to the *Centers for Medicare and Medicaid Services (CMS)*, national health expenditures as a percentage of the U.S. Gross Domestic Product (GDP) totaled 5% in 1965, but are expected to total 20% of GDP by the middle of this decade[4]. Although spending on cancer care comprises only 5% of the overall health care budget, these costs continue to rise at a pace more rapid than any other area of health care[5]. National cancer expenditures are projected to increase from 125 billion USD in 2010 to 173 billion USD in 2020[5]. Additionally, costs of oncology therapeutics and supportive care drugs have grown to reach 107 billion USD globally in 2015, an increase of 11.5% over 2014 (on a constant dollar basis) and up from 84 billion USD in 2010, as measured at invoice price levels. Annual global growth in the oncology drug market is expected to be 7.5 – 10.5% through 2020, reaching 150 billion USD[6].

Based on the above, on February 12th, 2016, during his speech to the Union States, President Barak Obama launched the so called "Moon Shot Cancer Program", declaring the following: "*For the loved ones we have lost, for the family we can still save let's make America the Country that cures cancer once*

and for all". President Obama allocated 1 billion USD to the program, asked Vice President Joe Biden to take the lead (J. Biden lost his 46 years old son for brain cancer), and fixed 2025 as deadline to reach this ambitious objective.

Rejuvenated by the "Moon Shot Program", research on prevention, early diagnosis, and development of novel and efficacious cancer treatments has become a top priority for the U.S. health care system (as well as for the health care systems worldwide) and indeed a tremendous progress has been achieved so far.

Trying to summarize the state of the art of the armamentarium of strategies put in place to fight cancer is out of the scope of this introduction. Since my experimental PhD program aimed at investigating the potential of therapeutic cancer vaccines, I will briefly focus on cancer immunotherapy, a field that has made a tremendous progress over the last few years and is expecting to revolutionize the way cancer patients can be treated.

1.1 Cancer Immunotherapy

For most of the twentieth century, the ability of the immune system to recognize and eradicate cancer was doubted by the vast majority of medical oncologists. Prevailing wisdom postulated that tumorigenesis and disease progression could not occur in the face of an immune response; thus, a diagnosis of cancer meant a priori that such antitumor responses had failed. Furthermore, the notion that immune responses may in fact prevent the occurrence of clinically manifested disease could not be proven unequivocally, as still remains the case today. However, several anecdotal case studies documented evidence of unexplained spontaneous tumor regressions in cancer patients, providing support for the idea that antitumor immune responses may play a critical role in some patients.

The critical relationship between immune function and cancer was first proposed by Rudolf Virchow 150 years ago when he observed the prevalence of leukocytes in tumors. A few decades later, William Coley, a medical doctor who received his license to practice medicine in New York in 1890, made the observation that occasional patients with inoperable cancers had remissions of their tumors during life-threatening infections such as severe erysipelas

(*Streptococcus pyogenes*)[7]. Thus, in 1891, he intentionally infected a patient with a large inoperable neck tumor with *S. pyogenes*. After a life-threatening systemic infection, the patient recovered and his tumor showed marked regression. In 1893, Coley produced extracts of cultures of *S. pyogenes* and *Bacillus prodigiosus* (an endotoxin producing organism) – known as the Coley's toxin. By 1914 Coley had treated over 500 patients, claiming to have induced over 150 remissions[8]. Additional evidence of connection between infections and tumor regressions has come from *Bacillus Calmette–Guérin* (BCG), a tuberculosis vaccine preparation consisting of attenuated *Mycobacterium*, with this treatment being used to the present day as the most effective therapy to prevent recurrence against superficial bladder cancer, presumably by activating an antitumor immune response[7].

The most convincing evidence for the existence of antitumor immunity came in the late 1980s from clinical trials which showed that some metastatic melanoma and renal cell carcinoma patients experienced dramatic tumor regressions in response to treatment with the cytokine interleukin (IL)-2, a potent activator of T cells[7]. Approximately 15% of patients had objective responses after IL-2 treatment, and half of these went on to be completely cured[9]. These results led the US Food and Drug Administration (FDA) to approve IL-2 in the late 1990s as the first *bona fide* immunotherapy for the treatment of cancer patients. They also inspired several research studies over the past two decades to develop alternative immunotherapies with better safety and efficacy and to improve understanding of IL-2 mechanism of action[9].

Today, there remains little doubt that the immune system has the inherent capacity to recognize and eradicate cancer and this awareness has triggered an intense research activity aiming at exploiting the intrinsic capacity of our own immune defenses to fight cancer. Tumors arise through a combination of genetic and epigenetic changes that generate "foreign" antigens, the so-called neoantigens, which make most neoplastic cells detectable by the immune system[10]. Unfortunately, some neoplastic cells can manage to escape immune recognition, developing mechanisms of immune evasion, induction of tolerance, and systemic disruption of T cell signaling[10]. Moreover, immune recognition of malignant cells inevitably triggers a selective pressure favoring the outgrowth of

less immunogenic and more apoptosis-resistant clones[10]. Aim of immunotherapy is to apply *ex vivo* and *in vivo* strategies to invigorate the immune system and make it capable of recognizing and destroy tumors that otherwise have successfully escaped natural immune surveillance.

The most promising immunotherapy strategies already approved or under intense clinical investigation are briefly described below.

1.1.1 Monoclonal antibodies (mAbs) therapy

Anti-cancer monoclonal antibodies (mAbs) are drugs designed to bind to specific targets on cancer cells and destroy them through four distinct mechanisms of action: (i) impairment of cell growth signaling, by binding soluble mediators and inhibiting their interaction with cognate receptors, or by working as agonist/antagonist of surface receptors; (ii) stimulation of antibody dependent cellular cytotoxicity (ADCC), mostly mediated by the recognition of antibody binding to tumor cells through Fc receptor engagement of Natural Killer (NK) cells and macrophages; (iii) activation of the complement dependent cytotoxicity (CDC), also mediated by antibody binding to tumor cells: (iv) induction of adaptive immunity against tumor-specific and tumor associated antigens. This mechanism is triggered by the release of cancer antigens from cells killed by CDC and ADCC.

The past two decades have seen the approval of several mAbs (Table I) that now are part of the standard clinical practice. For instance, mAbs targeting the

Table 1 Monoclonal antibodies approved for clinical use in oncology

Antibody name	Target	Antibody format	Application
Cetuximab	EGFR	Chimeric	Colorectal, breast and lung cancer
Panitumumab	EGFR	Human	Colorectal cancer
Nimotuzumab	EGFR	Humanized	Head and neck cancer
Rituximab	CD20	Chimeric	Non-Hodgkin lymphoma
Trastuzumab	HER2	Humanized	Breast cancer
Alemtuzumab	CD52	Humanized	Chronic lymphocytic leukemia
Bevacizumab	VEGFA	Humanized	Colorectal and lung cancer
Ofatumumab	CD20	Human	Chronic lymphocytic leukemia
Ipilimumab	CTLA-4	Human	Metastatic melanoma
Pertuzumab	HER2	Humanized	Breast cancer
Denosumab	RANK Ligand	Human	Solid tumor bony metastases
Brentuximab vedotin	CD30	Chimeric	Hodgkin's or systemic anaplastic large cell lymphoma
Gemtuzumabozogamicin	CD33	Humanized	Acute myelogenous leukemia
90Y-Ibritumomab tiuxetan	CD20	Mouse	Low grade or transformed B cell non-Hodgkin's lymphoma
Tositumomaband 131I-tositumomab	CD20	Mouse	Lymphoma

Table I. Taken from: ***Simpson, A. et al. Monoclonal antibodies for the therapy of cancer. BMC Proc.8, O6 (2014)***[11].

extracellular domain of the Epidermal Growth Factor Receptor (EGFR) are *Cetuximab* to treat colon and head and neck cancers and *Panitumumab* for colon cancer. Furthermore, *Trastuzumab* is an antibody targeting Human Epidermal Growth Factor Receptor 2 (HER2), expressed in approximately 15-30% of breast cancers and associated with a poor prognosis[12]. Finally, *Bevacizumab* is a mAb directed against vascular endothelial growth factor A (VEGFA) and has been approved for treatment of colorectal cancer (CRC), glioblastoma, cervical cancer, lung cancer, renal cell cancer, breast cancer and ovarian cancer[12]. In addition to the direct, modulatory effects on signal transduction, ADCC mediated through engagement of NK and macrophages, also contributes to the activity of these antibodies.

In addition to "naked" mAbs, antibody-drug conjugates (ADC) have also been developed. These antibodies have been conjugated to cell toxins using different conjugation chemistries. Thanks to the ability of such antibodies of being internalized upon binding to cancer cells, they release the toxic payload inside the cells thus promoting specific cell killing. In 2011 *Brentuximab vedotin* (*Adcetris*®), received FDA approval for the treatment of patients with Hodgkin lymphoma. *Brentuximab vedotin* is comprised of an anti-CD30 chimeric antibody attached to monomethyl auristatin E (MMAE) via a protease-cleavable dipeptide linker. This approval was based on the pivotal Phase II study of 102 patients with an overall objective response rate (ORR) of 75% with complete remission (CR) in 34% of patients. The median duration of response for those patients in CR was 20.5 months. More recently, *Brentuximab vedotin* received additional FDA approval for patients with unfavorable-risk relapsed or primary refractory classic Hodgkin's lymphoma who have undergone autologous stem-cell transplantation. The AETHERA Phase III trial noted an impressive median progression-free survival (PFS) improvement of 42.9 months in the *Brentuximab vedotin* group compared with 24.1 months in the placebo treated group[13].

The second ADC to receive FDA approval in solid tumors is *ado-Trastuzumab emtansine* (*T-DM1*, *KadcylaTM*). *T-DM1* was approved for use as a single agent for the treatment of patients with HER2 positive metastatic breast cancer (MBC) who previously received *Trastuzumab* and *taxane*, separately or in

combination[13]. The overall response rate in this patient population was 34.5%, with clinical benefit seen in 48.2% of patients. FDA approval for *T-DM1* was granted after the successful 991 patient Phase III EMILIA trial in which an overall progression-free survival of 9.6 months with *T-DM1* versus 6.4 months with lapatinib plus capecitabine was observed. Research into novel ADCs has considerable momentum, with over 100 open clinical trials (clinicaltrials.gov) currently exploring ADCs against novel antigen targets in cancer patients[13].

1.1.2 Immune check point inhibitors

The use of monoclonal antibodies to inhibit the natural down-regulation of T cell activation is revolutionizing the current approaches to cancer therapy. Not surprisingly, the prestigious journal *Science* elected an anti- cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) antibody as “molecular of the year” in 2013.

One of the critical steps of immune response is the activation of T cells which recognize, through their T cell receptor (TCR), cognate antigens associated to the major histocompatibility complex (MHC) of antigen-presenting cells (APCs). If TCR-antigen-MHC recognition occurs concomitantly with the binding of CD28 (on T cells) and B7 (on APCs), a full T cell activation occurs (Figure 1). T cell activation is temporally regulated to avoid that an over-activation of T cells could ultimately damage healthy tissues and cause autoimmunity[14]. The regulation of T cell activation is mediated by a set of regulatory molecules most of which up-regulated on the surface of activated T cells. For instance, CTLA-4 receptor is expressed on activated effector T cells and regulatory T cells (Tregs) and it inhibits proliferation of, and IL-2 secretion by, T cells by competing with CD28 for B7 interaction[14]. Preventing CTLA-4 binding to B7 using anti-CTLA-4 mAbs has shown to pleiotropically fire T cell activation and potently inhibit tumor growth and proliferation in both preclinical and clinical settings. These impressive pieces of data led to the FDA approval as a first line therapy for metastatic melanoma patients of *Ipilimumab,* a mAb directed against CTLA-4[15]. Pooled data from clinical trials of *Ipilimumab* confirmed durable clinical responses, depicted by a plateau in the survival curve beginning around year 3, that lasted 10 years or more in a subset of approximately 21% of

patients who would otherwise be destined to death[16]. In 2015, *Ipilimumab* was also approved by the FDA as adjuvant therapy for locally advanced melanoma.

The downside of the use of immune checkpoint inhibitors such as anti-CTLA-4 mAbs is that the uncontrolled stimulation of immune responses is usually accompanied by significant immune-related toxicities. Although such toxicity can be partially controlled by using systemic steroid therapy[14], the treatment usually has to be interrupted after a few months. Therefore, the development of strategies aimed at attenuating the immune toxicity of checkpoint inhibitors represents an area of intense research.

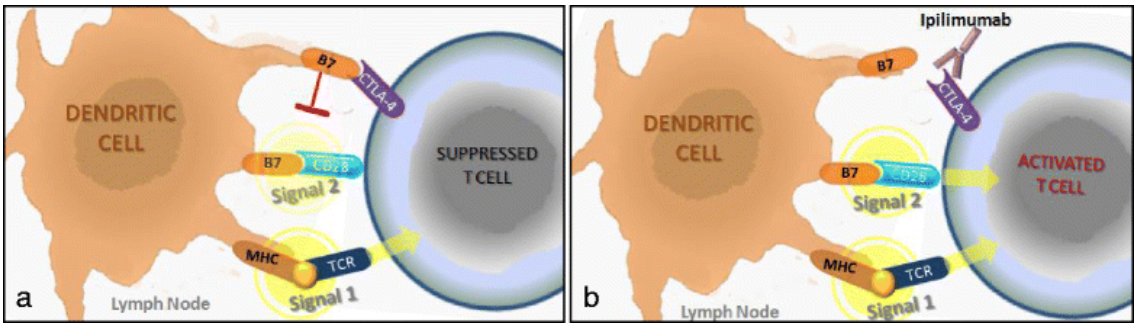


Figure 1. *Taken from:* ***Farkona, S., Diamandis, E. P. &Blasutig, I. M. Cancer immunotherapy: the beginning of the end of cancer? BMC Med. 14, 73 (2016)[10].*** *T cell activation. A) Both immunological signal 1 (T cell receptor (TCR) recognition of antigens) and immunological signal 2 (stimulation of CD28 by B7 costimulatory molecules) are required for T cell activation in the lymph node. The interaction between the CTLA-4 receptor and B7 expressed on T cells and APCs, respectively, prevents T cells from becoming fully activated by blocking immunologic signal 2. B) Antibodies that block the CTLA-4 pathway (e.g. Ipilimumab) permit T cell activation by derepressing signaling by CD28.*

Another checkpoint receptor expressed by activated T cells is programed death 1 (PD-1), expressed by antigen-stimulated T cells, which inhibits T cell proliferation, cytokine release and cytotoxicity. PD-1 fires T cell inhibition by binding to its cognate receptor PD-L1, which is up-regulated as a response to cytokine release by effector T cells on different cells, including T cells and tumor cells. Similarly to anti-CTLA-4 mAbs, anti-PD1 mAbs promote antitumor immune response. Alternatively, the inhibition of effector T cells can be prevented by using anti-PD-L1 antibodies that avoid PD-1/PD-L1 interaction (Figure 2). A large number of clinical trials have shown that antibodies blocking the PD-1/PD-L1 axis induce durable anti-tumor responses in many tumors[14,17]. This has led to the approval of two anti-PD1 antibodies (*pembrolizumab* and *nivolumab*) and one anti-PD-L1 antibody (*atezolimuma*b) for the treatment of

advanced melanoma, non-small-cell lung cancer (NSCLC), renal cell carcinoma, head and neck squamous carcinoma, Hodgkin's lymphoma, and bladder cancer[18].

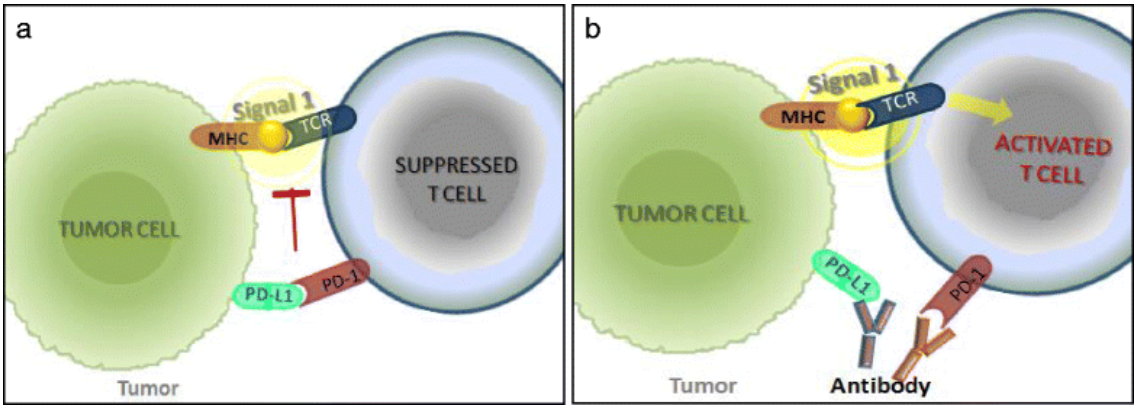


***Figure 2**. Taken from: **Farkona, S., Diamandis, E. P. &Blasutig, I. M. Cancer immunotherapy: the beginning of the end of cancer? BMC Med. 14, 73 (2016)[10].** T cell activation in the tumor milieu. A) PD1 receptor is an inhibitory receptor expressed by antigen- stimulated T cells. Interactions between PD1 and its ligand, PD-L1, expressed in many tumors activate signaling pathways that inhibit T-cell activity and thus block the antitumor immune response. B) Antibodies targeting PD1 or PD-L1 block the PD1 pathway and reactivate T cell activity.*

Currently there are over ten anti-PD-1 and anti-PD-L1 antibodies in various stages of clinical testing in many different tumor types. Interestingly, there have been thousands of patients receiving PD-1 blockade therapy thus far, with similar immune related toxicities as observed for anti-CTLA-4 but with generally lower frequency, possibly because the PD-1 and PD-L1 checkpoint may act later in the T cell response, resulting in a more restricted T cell reactivity toward tumor cells, with the majority of patients tolerating treatment well. Due to the non-overlapping mechanism of action of anti-CTLA-4 and anti-PD1 antibodies, clinical testing of the combination of these two classes of checkpoint inhibitors showed improved clinical response (up to 60%) in melanoma[19]. The combination of CTLA-4 and PD-1 and PD-L1 checkpoint blockade has been approved as front line therapy for advanced melanoma patients and is being tested in other tumor types with different dose levels and intervals of anti-CTLA-4 to reduce toxicity[14].

The success of anti-CTLA-4 and PD-1/PD-L1 mAbs prompted the research of other immune checkpoint pathways as targets for new immunotherapies[10]. For instance, the T cell immunoglobulin and mucin domain containing 3 (TIM-3) and lymphocyte activation gene 3 (LAG-3) are being studied. TIM-3, suppresses

effector T cell activation and is co-expressed with PD1 on CD8 tumor infiltrating lymphocytes (TILs)[10]. LAG-3 acts by binding to MHC molecules and also inhibits T cell activation and proliferation[20]. LAG-3 is co-expressed with PD-1 on T cells, making it a suitable candidate for a combinatorial approach with anti-PD-1 agents. Antibodies against TIM-3 and LAG-3 are under clinical investigation, showing encouraging efficacy.

Several other targets of host immunity are currently being evaluated in the pre-clinical and clinical settings, including inhibitory (IDO1, B7-H3, B7-H4, VISTA, ICOS, KIR and TIGIT) and stimulatory (OX40, 4-1BB and GITR) molecules[21].

1.1.3 Adoptive cell transfer (ACT) of T cells

The exploitation of adoptive transfer of T cells stems from the observation that cancer patients do have naturally induced anti-tumor T cells. However, they are not effective in preventing tumor growth since their number is too limited. Since technologies exist to isolate and expand T cells *in vitro*, the rationale of T cell adoptive transfer is to artificially enhance the population of tumor-specific T cells to a level that is therapeutically efficacious. Therefore, tumor-specific lymphocytes are first isolated from patients' peripheral blood, tumor-draining lymph nodes or tumor tissues, expanded *ex vivo*, and reinfused back into the patient[22]. Indeed, over the last two decades, autologous T cell therapies have demonstrated their potential by inducing dramatic clinical responses[10]. Prior to reinfusion, usually lympho-depletion is applied to patients and this procedure has resulted in durable, complete regression of melanoma[23]. In a pilot study at NCI cancer regressions by RECIST criteria (Response Evaluation Criteria in Solid Tumors) were seen in 21 out of 43 patients (49%), including 5 patients (12%) who underwent complete cancer regression[24]. When 200 or 1200 centigray (cGy;1 Gy = 100 rads) total-body irradiation (TBI) was added to the preparative regimen in pilot trials of 25 patients each, the ORR of 52 and 72% were seen, including 20 and 40% complete regressions[24]. Twenty of the 93 patients (22%) in these trials had complete regressions, and 19 (20%) have not experienced recurrences at follow-up times of 5 to 10 years and are probably cured. The observation that melanoma TILs can mediate durable, complete, and probably curative cancer regression in patients with metastatic melanoma

has raised considerable interest in the possible use of TILs for the treatment of multiple cancer types.

Adoptive transfer of genetically modified T cells is an effective alternative to classical ACT. There are two main T cell engineering platforms (Figure 3): (i) genetic manipulation of autologous T cells with TCRα and ß chains conferring high specificity for selected tumor epitopes, and (ii) T cell manipulation with chimeric antigen receptors (CARs). CARs are fusion proteins constituted by three domains: (1) an extracellular binding domain having specificity for the target tumor cells (usually single chain monoclonal antibodies recognizing surface exposed cancer antigens are used), (2) a transmembrane domain, and (3) a cytoplasmic domain firing T cell activation upon CAR binding to target cell. CARs offer two important advantages over classical adoptive T cell transfer. First, they carry binding modules with high affinity which bypass the fundamental issue of central tolerance. Second, being not MHC-restricted and processing-independent, CAR-based approaches are insensitive to MHC down-regulation and to altered processing escape mechanisms. The first successful clinical application of anti-CD19 CAR gene therapy in humans was reported in 2010[24]. Administration of autologous cells expressing the anti-CD19 CAR to a patient with refractory lymphoma resulted in cancer regression in a patient who remained progression-free after two cycles of treatment ongoing 4 years after treatment. Multiple groups have now shown the effectiveness of ACT targeting CD19 in patients with follicular lymphoma, large-cell lymphomas, chronic lymphocytic leukemia, and acute lymphocytic leukemia. These impressive results led to a large number of clinical trials of CAR T cells aiming at multiple hematological antigens, such as CD20, CD22 also frequently expressed in non-Hodgkin lymphoma and B cell acute Lymphoblastic Leukemia (B-ALL)[24].

Refinements of this strategy exploit T cells which carry an endogenous TCR specific for a strong immunogen and are genetically engineered to co-express a chimeric antigen receptor specific for a cancer antigen[25]. This dual-specificity of T cells has shown in preclinical studies anti-tumor reactivity upon allogeneic immunization. Phase I clinical study has demonstrated that dual-specific alloreactive CARs could be applied safely to ovarian cancer patients[26].

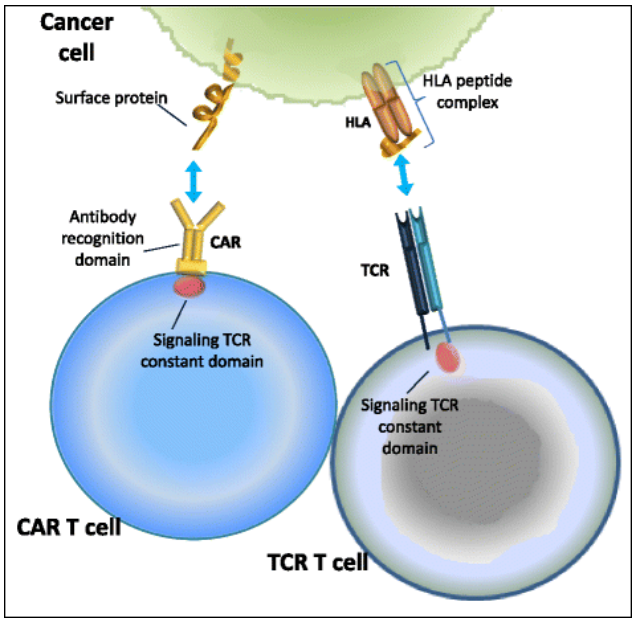


Figure 3. *Taken from:* ***Farkona, S., Diamandis, E. P. &Blasutig, I. M. Cancer immunotherapy: the beginning of the end of cancer? BMC Med. 14, 73 (2016)[10].*** *Genetic T cell engineering for the improvement and broadening of TIL therapy. CARs consist of an Ig variable extracellular domain fused to a TCR constant domain. The engineered T cells obtain the antigen- recognition properties of antibodies and thus are targeted against any potential cell surface target antigen. The expression of the TCR confers the engineered T cell with the antigen specificity of the transferred TCR. TIL therapy with TCRs is feasible for patients whose tumor harbors the HLA allele and expresses the target antigen recognized by the TCR.*

The biggest challenge facing the field of ACT is the identification of target tumor antigens that are not expressed by normal tissues, both to maximize specificity and efficacy and to minimize toxicity. A commonly seen toxicity in ACT therapy is cytokine release syndrome, which can be life-threatening and requires prompt management with steroids and IL-6 receptor antibody (*tocilizumab*)[14].

1.1.4 Adoptive transfer of Dendritic Cells (DCs)

Dendritic cells (DCs) play a crucial role in protecting the body from foreign antigens and form a link between the innate and adaptive immune system. Upon encountering foreign antigens, DCs act as sentinels of the innate immune response by releasing activating cytokines. As orchestrators of the adaptive immune response, DCs take up, process and present antigens on their cell surface to T-cells and B-cells, thereby activating naïve effector and memory immune cells or maintaining tolerance against self-antigens[27]. DCs are described to be the most potent endogenous activators of *de novo* T-cell and B-cell responses, highlighting their vaccine potential in eliciting potent anti-tumor immune responses[27].

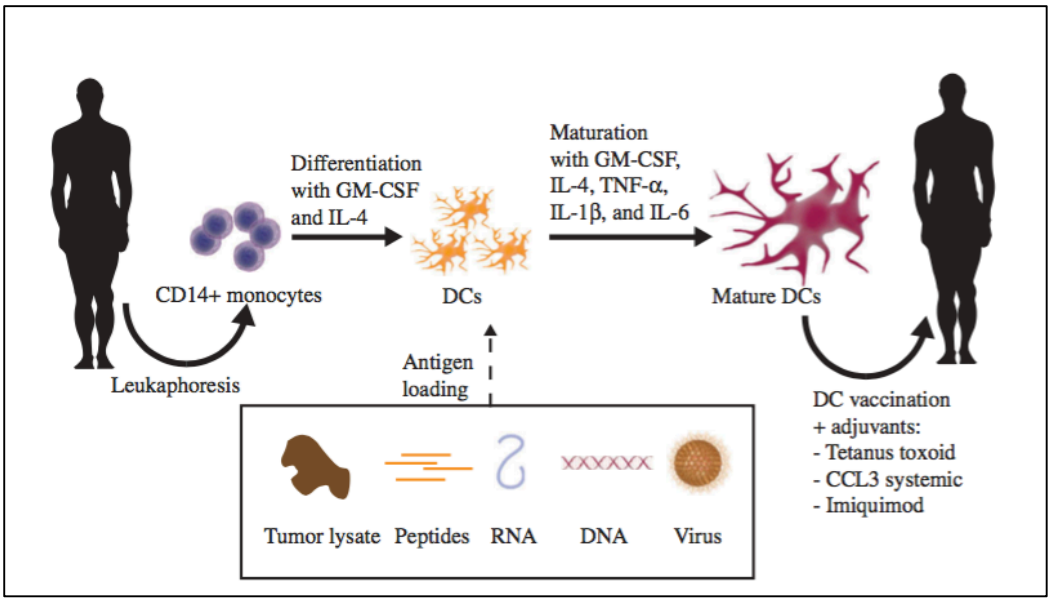


Figure 4. *Taken from:* ***Schaller, T. H. & Sampson, J. H. Advances and challenges: dendritic cell vaccination strategies for glioblastoma. Expert Rev. Vaccines584, 14760584.2016.1218762 (2016).***[27] *DCs for immunotherapy are generated in vitro using CD14+ monocytes isolated from patient PBMCs. Monocytes are typically differentiated into immature DCs by incubating with GM-CSF and IL-4 for a period of 5–7 days. DCs are subsequently matured in a cytokine cocktail for 16–20 hours and loaded with tumor antigen. DCs can be loaded with various formats of tumor antigen, including peptides, tumor lysate, DNA, and RNA. Finally, the DCs are injected back into the patient where they travel to vaccine-draining lymph nodes to elicit a tumor-specific immune response. Injection with adjuvants such as the tetanus toxoid can be used to increase DC migration to the lymph nodes and augment vaccine efficacy.*

The most common approach to generate clinical-grade DCs *in vitro* makes use of isolated CD14+ monocytes from patient peripheral blood mononuclear cells (PBMCs) (Figure 4). Over a period of 5–7 days, monocytes are differentiated into immature DCs by culturing with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. DCs are subsequently matured in a cytokine cocktail for 16–20 hours and loaded with tumor antigen. DCs can be loaded with various formats of tumor antigen, including peptides, tumor lysate, DNA, and RNA. Finally, the DCs are injected back into the patient where they travel to vaccine-draining lymph nodes to elicit a tumor-specific immune response. Injection with adjuvants such as the tetanus toxoid can be used to increase DC migration to the lymph nodes and augment vaccine efficacy[27].

Sipuleucel-T (*Provenge*) has been the first DC-based cancer vaccine approved in the U.S. and despite its relatively modest efficacy it is universally cited as the first demonstration of the potential of cancer immunotherapy in humans. The vaccine has been approved for castration-resistant prostate cancer patients and

is based on DCs partially purified from the blood of patients as described above and stimulated with a fusion protein consisting in prostatic acid phosphatase (PAP) linked to GM-CSF. Stimulated DCs are subsequently injected back into the patients. The vaccine has been approved on the basis of an objective increased median survival of 4.5 months[27].

Adoptive transfer of DCs is under intense investigation. A recent work by Anguille et al[28] reviews a number of published clinical trials. Overall, DC vaccination has clear objective responses even if relatively modest and often not dissimilar from other therapies. For instance, 8.5% of melanoma patients receiving DC therapy achieved an objective response, an efficacy similar to *dacarbazine* (the standard chemotherapeutic drug for treatment of melanoma) or to *Ipilimumab*, for which 5–15% of patients have an objective response. Moreover, 15.6% of patients with malignant glioma, and 11.5% of patients with advanced renal cell carcinoma 15.6% responded to DC therapy, a frequency comparable to what obtained with other immunotherapies such as IL-2[28].

There are number of challenges involving DC-based cancer vaccines including production issues necessary for uniformity in phenotype and activity (the realm of Good Manufacturing Practice—GMP). There are current questions as to what are the optimal means for generating DCs from precursors, as well as how to (or whether to) “mature” the cells, along with their preservation and re-growth after freezing. Additionally, the nature of the loaded antigenic material (source, format, single vs. multiple antigens, etc.) likely plays a critical role in the final vaccine formulation, but there is no true consensus[28]. As these DCs are usually injected back into a patient, migration of the DCs to lymph nodes is another area of concern.

1.1.5 Cytokines

Cytokines are molecular messengers made of interferons (INFs) and interleukins (ILs) that allow the cells of the immune system to communicate with each other to generate a coordinated, robust, self-limited response against a target antigen[29]. IFNα was approved for adjuvant therapy of stage III melanoma, Karposi sarcoma and several hematologic cancers. Previously mentioned IL-2 emerged to be a key cytokine in regulating T cells and NK

cellular survival, proliferation and differentiation and was approved by FDA for renal cell carcinoma in 1992 and later for stage III metastatic melanoma in 1998[30].

Nevertheless, IL-2 plays dual functions in the immune response both as a driver of effector lymphocyte responses, but also as a regulator or suppressor of effector lymphocyte responses at the same time by paradoxically also driving the expansion and suppressive function of CD4+ Foxp3+ Treg cells[30,31]. Main practical limits are comprehensive of serum short half-life which renders necessary high dose treatments resulting in toxicity: hypotension, pulmonary edema and heart toxicities[30]. IL-2 has also been shown to be applicable *ex vivo* for ACT by proliferation induction and cytolitic activity on Cytokine Induced Killer cells (CIK), NK as well as Lymphokine Activated Killer (LAK) and TILs cells and modulating T cell differentiation into Th1 or Th2 cells[32]. Alternative cytokines are IL-15 (with similar limitation in serum short half-life, augmentation of PD-1 expression and IL-10 production by CD8+ T cells, but with lower toxicity[33]) and IL-21, a relatively safe and better tolerated therapy but with dose-limiting side effects, liver toxicities and grade 3 or 4 granulocytopenia in some patients treated with some inhibitory effects in myeloid DCs[34]. Clinical studies are needed to define the safety and efficacy of IL-15 and IL-21 therapies[30]. Additionally, the extensive pleiotropism, redundancy of cytokine signaling and the dual function of many cytokines in both immune activation and immune suppression, poses significant challenges to our ability to achieve meaningful anti-tumor responses without also causing treatment-limiting toxicities. This dilemma is also well exemplified by the low response rates to notorious toxicities of IL-2. Engineered version of cytokine with reduced toxicity, such as IL-2-like cytokines and “superkine” can be exploited to circumvent main adverse effects and are under preclinical developments[35]. Otherwise a new approach uses antibody-cytokine fusion proteins or “immunocytokines” which allow the direct delivery of cytokines to the site of disease: this would reduce systemic toxicity, improve half-life and enhance immunomodulatory effects. To date, most immune-cytokines are based on IL-2a and many are under clinical development[35].

1.1.6 Cancer vaccines

According to the cancer immunoediting hypothesis, proposed by Schreiber[36], three main phases in recognition of cancer cells by our immune system take place: elimination, equilibrium, and escape. In particular, in the elimination phase, cells of the innate and adaptive immune response may eradicate the developing tumor and protect the host from tumor formation. If the elimination process is not successful, the tumor cells may enter the equilibrium phase and be immunologically shaped by immune "editors" to produce new populations of tumor variants. These variants may eventually evade the immune system and become clinically detectable in the escape phase[37].

Thus, to be effective, cancer vaccines must stimulate a robust tumor-specific immune response against the right targets such that the elimination phase prevails and cancer cells do not have the time to enter the equilibrium and escape phases. Whether or not cancer vaccines could ever be so effective is a question which has been vigorously debated over the last few decades. Indeed, while promising results have been reported by several groups in the preclinical settings, the clinical results have been so far disappointing. A cumulative analysis published in 2011 of several vaccine trials run from 2004 to 2009 reports that only 3.6% of the patients have had an objective benefit from vaccination[38].

Similar to all vaccines, cancer vaccine formulations must include (1) specific antigens, (2) adjuvants and (3) an antigen/adjuvant delivery system. In the absence of only one of these elements, vaccines would be unable of counteracting the inhibitory tumor microenvironment (containing Treg cells and aberrantly matured myeloid cells), and the highly mutable tumor targets (driving antigen loss and immune evasion).

1.1.6.1 Tumor antigens

Non-mutated, shared self-antigens constitute the majority of currently identified tumor associated antigens (TAAs) and can be identified as:(a) cancer-testis antigens (CTAs), such as NY-ESO-1 and MAGE-1 which are expressed in histologically different human tumors and, among normal tissues, in

spermatocytes/spermatogonia of the testis and, occasionally, in placenta. CTAs result from the reactivation of genes which are normally silent in adult tissues but are transcriptionally activated in different tumor histotypes. (b) Differentiation antigens, expressed by both tumors and normal differentiated cells from which the tumors arise for example, melanoma antigen and normal melanocytes (Gp100, MART-1, also known as Melan-A). (c) Self-antigens which are normally expressed by healthy cells but overexpressed by tumors, for example, mucin 1 (MUC1) and recently identified protocadherin FAT1[12,39]. The fact that normal cells express these antigens, even if in lower quantity, can trigger central and peripheral tolerance mechanisms but can also reach the threshold for T cell recognition, breaking the immunological tolerance and triggering an anticancer response[37].

Unique TAAs, on the other hand, are products of random somatic point mutations induced by physical or chemical carcinogens and therefore expressed uniquely by individual tumors and not by any normal tissue, representing the only true tumor-specific antigens. Such antigens characterize each single neoplasm and were shown to be diverse between tumors induced in the same animal or even in different tissue fragments from the same tumor nodule[37].

An important class of tumor antigens is made of tumor-specific neoantigens, which arise via genetic and epigenetic changes that alter amino acid coding sequences (non-synonymous somatic mutations). Some of these mutated peptides can be expressed, processed and presented on the cell surface, and subsequently become the targets of both CD4+ and CD8+ T cells. Because normal tissues do not possess these somatic mutations, neoantigen-specific T cells are not subject to central and peripheral tolerance, and also lack the ability to induce normal tissue destruction. As a result, neoantigens appear to represent ideal targets for T cell-based cancer immunotherapy. Importantly, neo-epitope-specific T cells have been found among TILs and when amplified *ex vivo* from tumor biopsies and introduced back into patients, TILs have shown anti-tumor activities[24]. Moreover, the impressive therapeutic effect of checkpoint inhibitor antibodies observed in a fraction of patients has been shown to correlate with the number of tumor-associated mutations[40–42]. On the basis of

these evidences, vaccines formulated with neo-epitopes have recently been devised and shown to be highly effective in preventing tumor growth in different preclinical and in recent clinical settings.

1.1.6.2 Cancer cell-derived exosomes: a potential source of TAAs

Exosomes are 30 to 100 nm diameter lipid bilayered membrane vesicles[43,44] composed of multiple proteins, DNA, mRNA, miRNA, long non-coding RNA[45]. Typically, exosomes form within cells, during endosome maturation, accumulating intraluminal vesicles (ILVs) by inward reverse budding of endosomal limiting membrane to form multi-vesicular endosomes (MVEs)[46] (Figure 5). During this process, cytosolic proteins, nucleic acids, and lipids are sorted into these small vesicles[47]. MVEs can either fuse with the lysosome and degrade their content, or fuse with the cellular membrane releasing formed ILVs as exosomes into the extracellular space[46,48]. Molecules inserted into the exosome membrane are thought to maintain the same orientation displayed in the whole cell (i.e., with extracellular domains exposed to the external milieu), thereby retaining their ability to bind cognate ligands[49]. Exosomes contain cell-specific payloads of proteins, lipids, and genetic material that are transported to other cells, where they alter cellular function and physiology[43]. The understanding of exosome biology has increased exponentially in recent years, leading to the creation of online databases such as Exocarta, EVpedia and Vesiclepedia[50–52], providing original research about exosomes with associated proteins, lipids and RNA from different sources[44].

Most cell types secrete exosomes, under both physiological and pathological conditions[53], especially tumor cells[54] as well as hematopoietic cells including reticulocytes[48,55], B and T lymphocytes, mast cells, macrophages, DCs and platelets. Furthermore, epithelial cells, fibroblasts, astrocytes and neurons produce exosomes and exosomes have been found in physiological fluids such as plasma/serum, urine, cerebrospinal fluid, breast milk, saliva, ascites and nasal secretion[52,56,57].

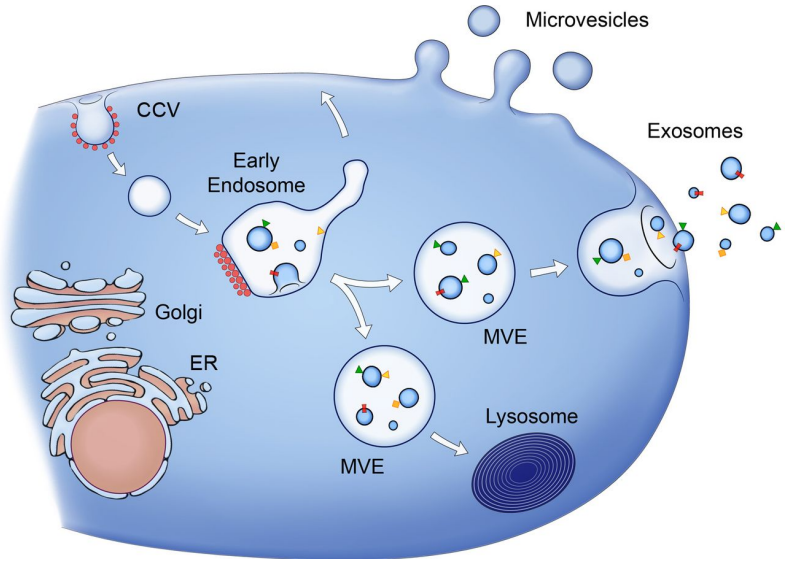


Figure 5. *Taken from:* ***Raposo, G. & Stoorvogel, W. Extracellular vesicles: Exosomes, microvesicles, and friends. Journal of Cell Biology 200, 373–383 (2013).***[57] *Schematic presentation of exosome's biogenesis and release by eukaryotic cells. Exosomes form within cells, by inward reverse budding of endosomal limiting membrane to form MVEs. MVEs that fuse with the cellular membrane release formed ILVs as exosomes into the extracellular space. Exosomes contain cell-specific payloads of proteins, lipids, and genetic material that are transported to other cells, where they alter cellular function and physiology. Microvesicles, instead bud directly from the plasma membrane. Red spots symbolize clathrin associated with vesicles at the plasma membrane (clathrin-coated vesicles [CCV]) or bilayered clathrin coats at endosomes. Membrane-associated and transmembrane proteins on vesicles are represented as triangles and rectangles, respectively.*

Interestingly, the release of exosomes is constitutive and particularly enriched in tumor microenvironment with respect to normal counterparts[58,59]. Indeed, tumor cells are avid exosome producers, and tumor-derived exosomes (TEXs) accumulate in cancer patient's plasma and may have special roles in cancer development.

TEXs have been reported to include many of the common exosomal proteins and to carry high amount of both known and unknown TAAs and neoantigens that, as said, are a "fingerprint" of the tumor they derive from[44,60]. It is generally accepted that multivalent vaccines based on antigen combination should be more effective than single subunit vaccines in the elicitation of a broad immune response[61]. These peculiarities make them good candidates for cancer vaccine multivalent delivery platform.

TEXs are associated with conflicting roles of immune stimulation as well as suppression[54]. Some studies have reported that TEXs released by cancer cells can induce apoptosis of T cells, impair DC differentiation, inhibit NK activity, and propagate immunosuppressive myeloid suppressor cells and Treg[54,60,62].

On the other hand, there is a rich number of publications and clinical studies clearly demonstrating that TEXs can also support immune response induction with important immunogenic properties. Tumor antigens, non-immunogenic when presented by tumor cells in a compromised suppressive tumor environment, induce Th, CTL and B cell responses and lead to a decrease in Treg, when presented by TEXs[63,64]. Exosomal heat shock protein (Hsp), which works as endogenous danger signal, promote NK activation and tumor cell lysis by granzyme B release[65]. Heat-stressed tumor cells produce exosomes that stimulate an effective tumor antigen specific CTL response. Increased immunogenicity of heat-stressed TEXs is further strengthened by data showing that exosomal intratumor injection efficiently attracts and activates DCs and T cells that inhibit tumor growth[66]. Exosomal release of HSP72 induced by radiation, increases CTL and NK activity and allows the expression of costimulatory molecule expression in DCs[67]. Furthermore, vaccination with staphylococcus enterotoxin A expressing TEXs significantly inhibits tumor growth by increasing IL-2 and IFNγ secretion, which promote Th, CTL and NK activation[68]. A recent study has shown the possibility to develop an efficient TEXs-adjuvant co-delivery system using genetically engineered TEXs containing endogenous tumor antigens and immunostimulatory CpG DNA. Such formulation has been reported to enhance tumor antigen presentation effect and to have a strong *in vivo* tumor inhibition skill[69]. Moreover, recent clinical trials of TEX-based cancer immunotherapy have demonstrated the feasibility, safety and efficacy of exosomes in a cancer vaccine formulation[70]. This study has been performed on patients with colorectal cancer stage III/IV. Exosomes were purified from ascites of each patient and administered together with GM-CSF[71]. Few patients did benefit from the combination and feasibility and safety were demonstrated[70].

Taking together all main features described above, preventive and/or therapeutic exploitation of TEXs appears promising, especially for the application as multivalent cancer vaccines[72], which does not require the direct identification or the specific purification of particular tumor antigens.

1.1.6.3 Adjuvants

Antigens alone are rarely sufficient to elicit protective immune responses. They need to be properly formulated with molecules, referred to as adjuvants, specifically selected to activate the appropriate effector mechanisms of immune response. To fight cancer both arms of the immune system, namely humoral and cell-mediated responses, play key roles. By binding to antigens exposed on the surface of cancer cells antibodies attack tumor cells via the four mechanisms described before (see section *1.1.1. Monoclonal antibodies (mAbs) therapy*). By contrast, cancer cells presenting antigens on their surface in the context of MHC I and MHC II can be destroyed by effector CD8+ and CD4+ T cells. The identification of adjuvants that can simultaneously potentiate the production of protective, antigen-specific, antibody and T cell responses represent a field of intense investigation.

The elucidation of the mechanisms of innate immunity and of how pathogen components trigger the first line of immune response and shape the effector mechanisms of adaptive immunity have substantially contributed to the discovery of new adjuvants. A few new adjuvants have already reached the market and an armamentarium of novel molecules have reached the clinical phases. Such molecules that are either components directly purified from pathogens or mimic structures and mechanisms of action of such components, are currently exploited in different formulations of cancer vaccines. A few examples are reported below.

Polynosinic-polycytylic acid (PolyI:C) stabilized with polylysine and carboxymethyl cellulose, (Poly ICLC or *Hiltonol*) is an adjuvant formulation which acts through the interaction to TLR3 receptor and RIG-1 receptor present in DCs and phagocytic cells. This formulation has shown anti-tumor efficacy when combined with different cancer antigens in many preclinical and some clinical studies[73,74].

TLR4 ligands are important adjuvants that are being tested for cancer treatments. Specifically, TLR4 recognizes Lipopolysaccharide (LPS) from Gram negative bacteria and a number of molecules mimicking LPS are being

produced. One of these, monophosphoryl lipid A (MPL), has been approved for microbial vaccines and is currently being tested in many clinical trials[75].

RNA (single stranded and double stranded) is another potent stimulator of innate immunity. It acts by binding to TLR receptors present in the vesicular compartment of immune cells. Several compounds mimicking RNA interaction to TLR7 are under evaluation, and FDA has already approved *Imiquimod*, a small molecule TLR7 agonist which is used to treat precancerous skin lesions (actinic keratosis)[73].

Promising immunotherapeutic adjuvants are synthetic oligonucleotides containing CpG motifs (CpG ODN). They act by binding to TLR9, which like TLR7, is expressed in the vesicular compartment of immune cells. Many clinical trials have been conducted in humans using CpG ODN and good elicitation of Th1 response, including antibody and antigen-specific T cell production, has been reported[73].

As previously mentioned, *Cytokines* such as IFN, IL-2 and GM-CSF can also be exploited as cancer vaccine adjuvants. GM-CSF can promote the recruitment and maturation of DCs together with the activation of macrophages, neutrophils, and NK cells[76]. Many cancer vaccine trials have incorporated GM-CSF. For instance, cancer vaccines constituted by EGFRvIII-derived peptide (*Rindopepimut*) and HER2-derived CD8+ T cell epitope peptide both have been formulated with GM-CSF and have reached Phase III for glioblastoma and HER2+ metastatic breast cancer[77], respectively.

Finally ligands of CLR (C-type receptor), RLR (retinoic acid inducible gene I – RIG1 – like receptors)and STING (stimulator interferon gene) are other potential adjuvants for cancer vaccines and their applicability has been analyzed in some preclinical and in very recent clinical studies[73].

1.1.6.4 Delivery systems

The third component of a vaccine is the delivery system. Antigens and adjuvants have to be efficiently taken up by APCs in order to properly activate adaptive immunity. It is well recognized that if incorporated in sufficiently large particles antigens and adjuvants can be co-delivered to the same APC and can

be better phagocytosed. Therefore, different antigens-adjuvants delivery strategies are being investigated.

Virosomes are spherical viral particles constituted by few components of viral capsid/envelop which, by virtue of the fact that do not contain nucleic acids, are incapable of replicating in the cells. Virosomes can be engineered to carry tumor associated/tumor specific antigens and/or adjuvants[73]. For instance, therapeutic cancer vaccine made of influenza virosomes containing Her/neu peptide was shown to be well tolerated and capable of inducing specific antibodies and cellular immune responses[78].

Liposomes are versatile, biocompatible and biodegradable synthetic phospholipidic vesicles. Several animal studies that use liposomes as adjuvants or delivery agents show superior anti-tumor efficacy over non-liposomal vaccines [73]. Safety and long lasting CD4+ and CD8+ T cell responses were demonstrated in clinical trials with follicular lymphoma patients[79].

Immune stimulating complexes are open cage like particles (~40nm) composed of *Quillaja saponins*, cholesterol and phospholipid. These can directly interact with DCs and allow CD4+ and CD8+ T cell responses. Their safety and clinical benefit have been evaluated in clinical trials with NY-ESO-1+ tumor patients [80]. Another saponin-based adjuvant is *QS-21,* shown to induce specific CTL and Th1 responses. Clinical trials for breast and prostate cancer treatments have shown antigen specific antibody responses and good toleration.

Aluminium phosphate or aluminium hydroxide are between the few approved adjuvants for human use for a variety of vaccines, but their application in cancer treatments is limited due to their incapacity to induce strong Th1 and cellular immune response[81]. High IgM and IgG antibody responses correlated with prolonged survival, were reported in a recent clinical trial in NSCLC patient against tumor associated ganglioside (NeuGcGM3)[73].

Water in oil emulsions adjuvants such as Incomplete Freund's adjuvant (IFA) – *Montanide*- have been widely used in the clinics for cancer vaccines[82]. Different forms such as ISA720 and ISA51 for melanoma and NSCLC treatments have been correlated with prolonged survival of treated patients[83,84].

Oil in water emulsions such as *MF59* is a squalene based oil-in water emulsion adjuvant. Approved for use in influenza vaccine, MF59 has a limited application for cancer treatment. Only in combination with Cytosine guanine dinucleotide oligodeoxynucleotides (CpG ODNs), MF59 has been shown to obtain effective responses for melanoma treatment in mouse models [85].

1.1.6.5 Bacterial Outer Membrane Vesicles: a vaccine delivery system with potent built-in adjuvanticity

More than 40 years ago, researchers made the observation that Gram-negative bacteria secrete Outer Membrane Vesicles (OMVs)[86]. They are closed spheroid particles of a heterogeneous size of 20-300 nm in diameter generated through a "budding out" mechanism of the bacterial outer membrane (Figure 6). The majority of their components are represented by LPS, glycerophospholipids, outer membrane proteins (Omp) and periplasmic proteins[86].

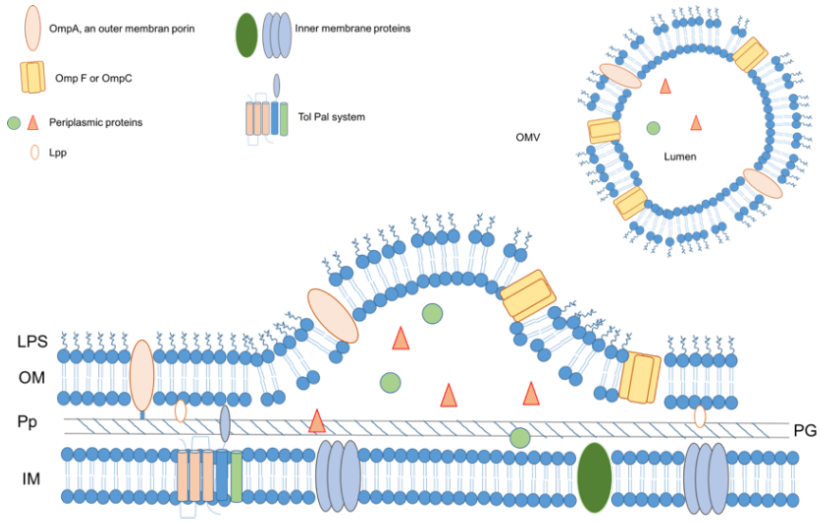


***Figure 6**. Schematic presentation of OMV's biogenesis. OMVs are generated through a "budding out" mechanism of the bacterial outer membrane. The majority of their components are represented by LPS, glycerophospholipids, Omp and periplasmic proteins. Proteins and lipids of the inner membrane and cytosolic content are excluded from OMVs. (Pp) periplasm; (OM) outer membrane; (PG) peptidoglycan; (IM) inner membrane (Lpp) Lipoproteins.*

As a specific secretory pathway, OMVs have a big number of functions, including inter and intra species cell-to-cell cross-talk, biofilm formation, genetic transformation, defence against host immune responses, toxin and virulence factor delivery to host cells. Similar to exosomes, OMVs interaction to host cells can occur by endocytosis from binding to host cell receptors or lipid rafts and by

fusion to host cell membrane, leading to the direct release of their content into the cytoplasm of the host cells[87].

From a biotechnological standpoint, OMVs are increasingly attracting researcher's interest as an emerging unique vaccine platform and in fact anti-Neisseria OMV-based vaccines have been developed for human use[88].

OMVs have three main features that make them particularly promising as vaccine platform.

First, they carry several Microbe Associated Molecular Patterns (MAMPs), including LPS, lipoproteins and peptidoglycan, which play key roles in stimulating innate immunity and promoting adaptive immune response. OMVs immunization elicits potent antibody responses against the OMV-associated proteins. Moreover, OMVs can elicit potent Th1-skewed immune responses as reported in a number of recent studies[89].

Second, the OMV protein content can be altered by applying genetic and synthetic biology manipulation of the OMV producing bacterium. An increasing number of studies has reported the possibility of decorating OMVs with heterologous proteins both in the lumen and exposed on the vesicle surface[90]. Kesty and Kuhen[91] were the firsts to show the delivery of proteins into the OMV compartment. Subsequently, several other authors have manipulated the OMV protein content by using different strategies[90]. In our laboratory different bacterial antigens were delivered to the lumen of *E. coli* vesicles by fusing their coding sequences to a leader peptide for secretion[89]. More recently (Fantappiè et al 2017[92]), we showed the successful expression of lipoproteins from *Neisseria meningitidis (Nm)* and *Aggregatibacter actinomycetemcomitans* on the surface of *E. coli*-derived OMVs and the possibility of exploiting them as delivery vehicles of heterologous polypeptides to the OMV surface.

Third, OMVs can be easily purified from the culture supernatant and the OMVs production process is scalable at industrial levels. Several mutations have been reported that promote the release of abundant quantities of OMVs in the culture supernatant and GMP production of OMVs with yields higher than 100 mg/L of culture has been reported[93].

1.1.6.6 Cancer vaccines: state of the art

Cancer vaccination comprises an array of approaches that seek to generate, amplify, or skew (or a combination thereof) antitumor immunity. A successful therapeutic cancer vaccine activates a cancer patient's immune system, resulting in eradication or long-term control of disease. Such a vaccine typically consists of a tumor antigen in an immunogenic formulation and activates tumor antigen–specific helper cells and/or CTLs and B cells. B cells secrete their specific antibodies to cause the lysis or phagocytosis of cells that display antigens they recognize. CTLs use their TCR to specifically recognize small cell-derived peptides presented on a cell's surface bound to MHC I class molecules. If the T cell is activated and its TCR binds a particular MHC/peptide complex, it will release cytotoxic molecules and cytokines that will kill the cell and stimulate activation of nearby immune cells. CD4+ Th cells can promote the activation of both B cells and CTLs. So, cancer vaccines cause the selective activation and proliferation of B and T cells that can recognize tumor cells, thus preparing them for their cancer-killing action.

Generally, cancer vaccines can be employed in the form of (i) peptide antigen vaccines, (ii) whole-cell vaccines, which encompass autologous, allogeneic cell vaccines (iii) nucleic acids that encode for tumor antigens and can stimulate innate immunity by sensitizing TLR and intracellular sensors virus based strategies.

1.1.6.6.1 Peptide based cancer vaccines

Peptide antigen vaccines can include synthetic or purified native moieties that are representative of the tumor cell antigens displayed by the target tumors. These antigens can be used to immunize patients and have been shown to generate an immune response capable of destroying cells in the body that display these antigens. These types of cancer vaccines are dependent upon knowing the major tumor cell markers/antigens, their structure, and, if peptides are generated, the important epitope(s) required to generate a tumor-specific immune response. Peptide antigen vaccines can be constructed from the most immunogenic or cancer specific epitopes, but since peptides are not sufficiently antigenic, they are typically accompanied by an adjuvant. Peptides formulated

in adjuvants (such as Montanide) with or without cytokines, such as GM-CSF and INFγ, or TLR agonists, have shown clinical benefit (partial responses, complete responses, and durable disease stabilization) in small and large scale clinical trials. Another strategy that has shown significant clinical efficacy is the use of synthetic peptides that are long enough to include multiple MHC class I and II epitopes. These 23-45 amino acid long peptides, delivered subcutaneously (s.c.), have been shown to be especially effective, possibly because of a more efficient processing and presentation, which leads to superior T cell activation[94].

1.1.6.6.2 Whole- cell-based cancer vaccines

The whole-cell vaccine approach encompasses the use of inactivated whole-tumor cells and/or whole-cell lysate as the vaccine. As such, these whole-cell vaccines present an array of TAAs to the patient's immune system. The approach of using whole-tumor cell as a vaccine eliminates the significant problem of having to identify the crucial antigen(s) for that cancer, most of which remain unknown. However, the approach of using whole-tumor cell is limited by the fact that antigens are diluted among a plethora of self-antigens, which results in poor cancer antigen uptake by APCs and inadequate antigen cross-presentation and T cell responses[95].

1.1.6.6.3 Nucleic acid-based cancer vaccines

Nucleic acid-based cancer vaccines are constituted by either DNA or RNA sequences encoding selected tumor antigens. The vaccines are delivered either as "naked" molecules (DNA) or formulated in appropriate delivery systems and allow for a patient's intracellular machinery to translate and process tumor antigens. These vaccines have the advantage of being relatively simple to produce and of having self-adjuvanticity properties, thanks to their capacity to stimulate different signaling pathways of innate immunity. In particular, RNA vaccines can activate the innate immune system by acting as TLR agonists for TLR7 and TLR8, and for this reason they are becoming a promising strategy for cancer vaccines[95].

1.1.6.6.4 Virus-based cancer vaccines

1.1.6.6.4.1Viral-based vectors

The rationale for using viruses as immunization vehicles is based on the phenomenon that viral infection often results in the presentation of MHC class I/II restricted, virus-specific peptides on infected cells. The viral vectors with low disease-causing potential and low intrinsic immunogenicity are engineered to encode TAAs or TAAs combined with immunomodulating molecules. An extensively evaluated viral-based vectors in cancer vaccine trials are from the poxviridae family, such as vaccinia, modified vaccinia strain Ankara (MVA), and the avipoxviruses (fowlpox and canarypox; ALVAC)[96]. Poxviruses have the ability to accommodate large or several transgene inserts. Poxvirus replication and transcription are restricted to the cytoplasm, which minimizes risk to the host of insertional mutagenesis. It is believed that induction of a local inflammatory response by the host TLRs and other properties of vaccinia or MVA contribute to the enhanced immune response reactive with inserted TAAs in preclinical studies. One promising viral cancer vaccine is *PROSTVAC* developed by Bavarian Nordic. This “off-the-shelf” platform consists of a replication-competent vaccinia priming vector and a replication-incompetent fowlpox-boosting vector. Each vector contains transgenes for PSA and three costimulatory molecules (CD80, CD54 and CD58) that are collectively designated TRICOM[96]. In double-blinded, placebo-controlled phase II trial, *PROSTVAC* improved median overall survival relative to the control vector (25.1 vs. 16.6 months, P =0.006)[96]. Similar improvement in the median overall survival was also observed in a second PROSTVAC single-arm phase II study. The pivotal phase III trial following these encouraging data from phase II studies are ongoing (NCT01322490).

1.1.6.6.4.2 Oncolytic viruses

Oncolytic viruses are included in viral strategies as one emerging class of therapeutic with the potential to act in synergy with novel immunotherapies to improve clinical outcomes. Naturally occurring or engineered oncolytic viruses act to replicate specifically and kill cancer cells without harming the normal tissues.

1.1.6.6.4.2.1 Herpes simplex virus

Herpes simplex virus type 1 (HSV-1) is an enveloped dsDNA virus with the ability to infect a wide variety of cell types, and to incorporate single or multiple transgenes. A genetically engineered oncolytic HSV-1 is *T-VEC (talimogen elaherparepvec, IMLYGIC*, formerly *OncoVEXGM-CSF*), encoding human GM-CSF for direct injection into accessible melanoma lesions was approved for melanoma by the FDA in the U.S. in 2015 and in Europe and Australia in 2016. The addition of GM-CSF promotes monocyte-to-dendritic cell differentiation, thereby facilitating antigen presentation on the surface of dendritic cells following viral-induced oncolysis. The phase III trial proved that local intralesional injections with T-VEC in advanced malignant melanoma patients can not only suppress the growth of injected tumors but also act systemically and prolong overall survival. The trial found an ORR of 26% and a complete clinical response in 11% of patients with stage IIIB-IV melanoma[97]. Many clinical trials using *T-VEC* are currently performed worldwide by the pharmaceutical company in order to expand its application.

In Japan, a phase II clinical trial of G47Δ (third-generation oncolytic HSV-1), is ongoing in glioblastoma patients (UMIN000015995). G47Δ was recently designated as a “Sakigake” “ahead of the world” breakthrough therapy drug in Japan. This new system by the Japanese government should provide G47Δ with priority reviews and a fast-track drug approval by the regulatory authorities.

1.1.6.6.4.2.2 Oncolytic Adenovirus

Recombinant adenovirus (Ad) is another system that can be used as carriers for genetic vaccination. Adenoviruses are easy to engineer and propagate to high yields for clinical use. They also have the advantage of transducing both dividing and non-dividing cells for high expression of transgenes. Indeed, adenoviruses are used extensively as cancer gene therapeutic agents. One strategy to create oncolytic adenoviruses (oAds) involves deleting the Ad E1A gene, which results in a virus more directed towards replication in transformed cells[98]. More recently, miRNA-controlled oAds have been developed to attenuate virulence in quiescent cells while still retaining their full lytic capacity in human-derived xenografts. Another popular strategy is to create

transcriptionally regulated versions of oAds harboring tissue-specific promoters that can drive key virus regulatory genes.

Closed to drug approval in North America and Europe is *CG0070*. This is an Ad5 adenovirus engineered so that the human E2F-1 promoter drives the E1A gene, and the human GM-CSF gene is inserted. E2F-1 is regulated by the retinoblastoma tumor suppressor protein (Rb), which is commonly mutated in bladder cancer, and a loss of Rb binding results in a transcriptionally active E2F-1.

1.1.6.6.4.2.3 Vaccinia virus

Similar strategies to those discussed above have been employed with vaccinia virus to direct virulence towards transformed cells. For instance, *JX-594 (pexastimogene devacirepvec, Pexa-Vec)* is a genetically engineered vaccinia virus that has a mutation in the TK gene, conferring cancer cell-selective replication, actually in phase III trial for advanced state hepatocellular carcinoma, and an insertion of the human GM-CSF gene, augmenting the antitumor immune response. The advantages of using vaccinia virus include intravenous (i.v.) stability for delivery, strong cytotoxicity and extensive safety experience as a live vaccine[99].

In addition, several other virus families have served as backbones for the development of oncolytic viruses. This list includes, but is not limited to, Paramyxoviruses (Newcastle Disease virus, Measles virus, Reovirus - Reolysin) and Rhabdoviruses (Vesicular Stomatitis virus, Maraba virus)[98].

All genetically engineered oncolytic viruses described are designed to enhance the induction of antitumor immunity that accompanies the oncolytic activity. For example, both *T-VEC* and G47Δ have a deletion in the a47 gene, the product of which inhibits the transporter associated with antigen presentation; therefore, cancer cells subjected to the oncolytic activities of these viruses are vulnerable to immune surveillance, and the processing by APCs is likely facilitated. Furthermore, the local intralesion injection of *T-VEC* has shown to act on remote lesions via induction of systemic antitumor immunity and prolonged survival.

Because an induction of specific antitumor immunity in the course of oncolytic activities is the common feature that plays an important role in presenting antitumor effects, a combination of oncolytic virus components with systemic administration of immune checkpoint inhibitor or ACT approaches are reasonable strategies to enhance the efficacy of oncolytic viruses. It would not be too early to say that oncolytic virus therapy is now established as an approach to treat cancer.

1.1.6.6.5 Mutanome and personalized cancer vaccines

Tumors contain a large number of mutations, ranging from the tens to hundreds of somatic nonsynonymous mutations (the mutanome), that are unique to the tumor relative to the normal cells. Causative “driver” mutations shared by a subpopulation of patients can sometimes be targeted by small molecule inhibitors, such as the BRAF V600E mutation[100,101]. However, in the vast majority of cancer types there are no highly penetrant mutations. Rather, 95% of the mutations in a patient tumor appear to be unique to that tumor[102]. Thus, mutations may make ideal therapeutic targets, provided there was, for an individual patient, a platform to identify the mutations in the patient’s tumor and an effective way to efficiently target them.

Therapeutic cancer vaccination, in which a patient’s immune system is taught to target cancer cells, represents a promising therapeutic modality. Existing cancer vaccines, including several in clinical trials, target antigens with tumor-specific expression. A key challenge is immune tolerance against self-proteins. Tumor specific mutation antigens, in contrast, are not subject to central tolerance mechanisms and in fact immune responses to mutation-derived epitopes have been demonstrated in cancer patients. Thus, the tumor mutanome offers a large number of potential vaccine targets and thanks to the “next-generation sequencing” (NGS) technology which allows rapid and inexpensive identification of all immunogenic mutations, the development of individualized vaccines is becoming a reality. To demonstrate this, Kreiter and co-workers[101] analyzed the mutations present in the murine B16F10 melanoma cell line system and designed a synthetic RNA vaccine encoding one specific mutation-derived CD4+ epitope. The vaccine, formulated in liposomes, was

used to immunize syngeneic C57bl/6 mice and the effect of immunization on tumor growth after challenge with B16F10 cell line was analyzed. A remarkable growth inhibition induced by immunization was achieved.

These findings have generated great enthusiasm for neo-epitope vaccines. Massive parallel DNA/RNA sequencing combined with advanced computational methods enable the high-throughput identification of all prevalent nonsynonymous mutations in a tumor sample, and provide the basis for antigen discovery focusing on the identification of neoantigens in patients.

Very recently, the first-in-human testing of such an approach has been conducted by Sahin and co-workers[103]. Non-synonymous mutations expressed by 13 patients with stage III and IV melanoma were identified by comparative exome and RNA sequencing of routine tumor biopsies and healthy blood cells. Ten selected mutations per patient were engineered into two synthetic RNAs, each encoding five linker-connected 27mer peptides with the mutation in position 14 (pentatope RNAs) and percutaneously injected into inguinal lymph nodes of each patient. All patients completed treatment with a maximum of 20 neo-epitope vaccine doses. The patients had a recent history of recurrent disease and a high risk of relapse. Comparison of documented cancer recurrences in treated patients before and after neo-epitope vaccination showed a significant reduction of cumulative recurrent metastatic events ($P < 0.0001$), translating into good progression-free survival.

A second milestone paper demonstrating the efficacy of neo-epitope based cancer vaccine has been very recently gained by Ott and coworkers[104]. In a phase I study patients with previously untreated high-risk melanoma (stage IIIB/C and IVM1a/b) were vaccinated after surgical resection with synthetic peptides covering several neo-epitopes in the presence of Hiltonol as adjuvant. The authors reported that immunization induced CD4+ and CD8+ T cells specific for 60% and 16% of the epitopes, respectively. Furthermore, of six vaccinated patients, four had no recurrence at 25 months post vaccination, and the two of them with recurring disease were treated with *Pembrolizumab* showing then complete tumor regression. These data provide a strong rationale

for further development of this approach, alone and in combination with checkpoint blockade or other immunotherapies.

2 AIM OF THE PROJECT

One of the main objectives of the Synthetic and Structural Vaccinology Unit (SSVU) I joined for my PhD experimental work is to test whether bacterial OMVs can be exploited as cancer vaccines in human patients.

To this aim, the overall strategy of SSVU can be summarized as follows:

1. Selection of cancer antigens expressed in murine cancer cell lines known to induce tumors in immunocompetent syngeneic mice.

2. Decoration of OMVs with selected antigens using innovative molecular and synthetic biology strategies.

3. Purification of antigen-carrying OMVs and analysis of their immunogenicity properties after administration to immunocompetent mice either alone or in combination.

4. Analysis of protective activities induced by immunization with OMVs carrying one or more antigens in mice challenged with tumor cells.

In the course of my experimental work I have partially contributed to 1) the selection of a few cancer antigens, 2) the construction of bacterial strains expressing the selected cancer antigens, 3) the purification of OMVs from engineered strains, 4) the analysis of the immune responses and protective activities of engineered OMVs administered to mice challenged with cancer cell lines. This work has so far brought to the publication of two papers (Fantappiè' et al. Molecular and Cellular Proteomics, 2017; Zerbini et al. Microbiol. Cell Factories, 2017) and to the submission of one additional paper (Grandi et al. Frontiers in Oncology, 2017), of which I am one of the authors.

Furthermore, I have dedicated a large part of my experimental activity, of which I have been directly responsible, to 1) the preparation of exosomes from murine cancer cell lines as a source of cancer antigens, 2) the analysis of exosome/OMV interaction, 3) the analysis of immune responses elicited by exosome/OMV combinations.

In this document, I will focus my attention to describe the data showing that OMVs can indeed induce protective activities when decorated with selected cancer antigens and that such protective activity is potentiated in the presence of multiple antigens. Furthermore, I will show that exosomes from CT26 cancer cell line induce specific immune responses when combined with OMVs and such responses appear to synergize with the protective activity of OMVs decorated with a CT26-specific cancer antigen.

3 RESULTS

3.1 Antigen selection

As pointed out in the “Aim of the Project”, in our laboratories we are addressing two main questions. First, we are interested to know whether OMVs decorated with well-known cancer-specific epitopes could induce epitope-specific immune responses and whether such responses could protect immunocompetent mice from the challenge with syngeneic cancer cell lines expressing the epitopes. Second, we want to investigate whether OMVs decorated with a combination of antigens expressed in the same cancer cell lines could result in a synergistic protective activity when mice are challenged with such cell lines.

Based on the above, we are focusing our attention on two murine cancer cell lines, B16F10EGFRvIII and CT26, which induce the formation of large tumors when injected s.c. in C57/bl/6 and BALB/c mice, respectively. We are selecting antigens specifically expressed in these cell lines, and we are testing their protective activity when formulated alone or in combination.

3.1.1 B16F10-specific cancer epitopes: EGFRvIII and M30 epitopes

As far as the B16F10 cell line is concerned, two peptide antigens, LEEKKGNYVVTDH and PSKPSFQEFVDWENVSPELNSTDQPFL, were selected.

LEEKKGNYVVTDH peptide (EGFRvIIIpep) belongs to EGFRvIII, a mutated form of the human epidermal growth factor receptor (EGFR), expressed on several tumors and associated to the expression of epithelial–mesenchymal transition (EMT) and cancer stem cell genes. EGFRvIII contains an in-frame deletion in the extracellular domain of EGFR, creating a novel antigenic epitope which is exquisitely tumor-specific[105]. Immunization with EGFRvIIIpep conjugated to Keyhole limpet hemocyanin (KLH) was shown to protect mice from the challenge of syngeneic cell lines stably transfected with human EGFRvIII. In particular, Heimberger and co-workers showed that the conjugated peptide formulated with GM-CSF protected C57bl/6 mice from both extracerebral and intracerebral challenge with B16F10 cells expressing human

EGFRvIII[106]. Based on these data the vaccine (*Rindopepimut*) for EGFRvIII-positive glioblastoma patients was proposed and tested in different trials.

As far as PSKPSFQEFVDWENVSPELNSTDQPFL peptide (B16-M30pep) is concerned, it was recently described by Kreiter and co-workers as a CD4+ T cell epitope expressed in the B16F10 cell line as a consequence of a mutation occurred in the *kif18b* gene[107]. Therefore, M30 is a B16F10-specific neo-epitope not expressed in the syngeneic healthy C57bl/6 mouse tissues. Interestingly, the authors showed that immunization with liposome-formulated synthetic RNA coding for B16-M30 induced robust T cell-mediated protection in C57bl/6 mice when challenged with B16F10 cells[107].

3.1.2 CT26-specific cancer antigens: FAT1 and CT26-derived exosomes

As far as CT26 cell line is concerned, the attention was focused on FAT1 and CT26-derived exosomes.

3.1.2.1 FAT1

FAT1 is a type 1 transmembrane protein carrying an extracellular region with 34 cadherin repeats and a cytoplasmic tail[108]. Alteration of FAT1 expression and function has been associated to several human cancers. In many tumors FAT1 acts as a tumor suppressor while in others FAT1 is up-regulated. It was recently reported that FAT1 is highly expressed on the surface of most early and late stage CRCs and in CRC liver metastases, while in healthy tissues FAT1 is either not expressed or its expression is confined to the cytoplasmic space. Moreover, a FAT1-specific monoclonal antibody (mAb198.3), which binds the surface of cancer cells within a 25 amino acid sequence of the cadherin domain 8 (IQVEATDKDLGPNGHVTYSIVTDTD) (D8 epitope), inhibits the growth of human colon cancer cell lines in xenograft mouse models[39]. Since in our laboratories we found that the mouse homologue of FAT1 (mFAT1), which shares 87.3% identity to human FAT1, is also over-expressed in CT26, we decided to decorate OMVs with the mouse D8 epitope and test the protective activity of D8-mFAT1 OMVs in BALB/c mice against CT26 cell line challenge.

3.1.2.2 CT26 exosomes

Cancer cells abundantly release exosomes (TEXs) and TEXs have been shown to carry TAAs and neoantigens specific of the tumor they derive from. As previously mentioned, the lists of proteins experimentally found to be associated to different preparations of exosomes, including exosomes from cancer cell lines, can be found in several databases available on line, such as Exocarta, EVpedia and Vesiclepedia. Therefore, exosomes represent a subcellular compartment enriched in tumor antigens potentially exploitable for multivalent vaccine development.

In consideration of our interest to test the protective synergistic effect of multiple tumor antigens when combined with OMVs, we decided to analyze whether the immunization of BALB/c mice with the combination of D8-mFAT1 OMVs/CT26-derived TEXs could elicit a protective immune response against CT26 challenge superior to the one observed with D8-mFAT1 OMVs alone. The presence of tumor antigens in CT26-derived exosomes was not experimentally defined in a systematic manner. However, we analyzed whether FAT1, which, as said above, is overexpressed in CT26, accumulated in TEXs. Indeed, Western Blot (WB) analysis of CT26 total cell extract and purified CT26-derived TEXs clearly confirmed the presence of mFAT1 antigen in TEXs (Figure 7). On the basis of this evidence and assuming CT26-derived TEXs could incorporate other TAAs with both specific B and T cell epitopes, an important part of my work has been dedicated in testing the exploitation of CT26 derived TEXs as cancer formulation component.

3.2 OMVs decoration with cancer epitopes

Selected epitopes were subsequently used to decorate OMVs. To this aim, epitopes were fused to two different proteins that in our laboratories have been shown to efficiently deliver foreign polypeptides to the OMV compartments.

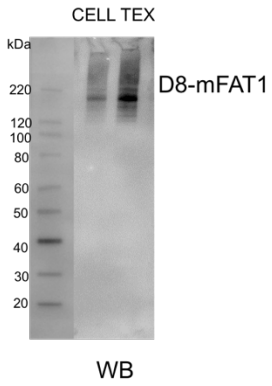


Figure 7. *Western Blot (WB) analysis of CT26 total extracts and CT26-derived TEXs. Samples corresponding to total cell extracts from CT26 cell line (CELL- 20 µg) and TEXs isolated from CT26 cells (TEX – 5 µg) were separated by SDS-PAGE. After protein transfer to nitrocellulose membrane, CELL and TEX were visualized using rabbit antibody against synthetic D8-mFAT1 peptide and peroxidase conjugated anti-rabbit immunoglobulins.*

In the first construct, the DNA sequence coding for three copies of the EGFRvIIIpep was ligated at the 3' end of the gene encoding the full length Nm-fHbp, generating the plasmid pET-Nm-fHbp-vIII. The plasmid expresses the chimera constituted by fHbp carrying three copies of the EGFRvIIIpep fused at its C-terminus.

The second construct is represented by the DNA sequence encoding Nm-fHbp and carrying at its 3' end three copies of M30 coding sequence followed by three copies EGFRvIIIpep (pET-Nm-fHbp-M30-vIII construct). The plasmid encodes a fusion protein in which fHbp is fused to three copies of M30 peptide followed by three copies of EGFRvIIIpep.

The third construct was characterized by three copies of the D8-mFAT1 coding sequence fused to the 3' end of MBP sequence forming the pET-MBP-D8-mFAT1 plasmid[89]. The construction details of plasmid pET-Nm-fHbp-M30-vIII and pET-MBP-D8-mFAT1 are reported in the Materials and Methods section.

The plasmids encoding the fusion proteins were used to transform the *E. coli* OMV-overproducing strain BL21(DE3)*ΔompA* and OMVs were isolated and purified. Purified vesicles were quality controlled by SDS-PAGE. As shown in Figure 8A, protein bands migrating with the expected molecular masses of the fusion proteins and not present in OMVs derived from BL21(DE3)*ΔompA* transformed with the "Empty" vector were clearly visible. Expression of EGFRvIIIpep in OMVs was confirmed by WB analysis (Figure 8B) and immune

Transmission Electron Microscopy (TEM). For TEM, OMVs were first incubated with rabbit anti-EGFRvIIIpep antibodies and subsequently with 5-nm gold-labeled anti-rabbit secondary antibody (Figure 8 C-D).

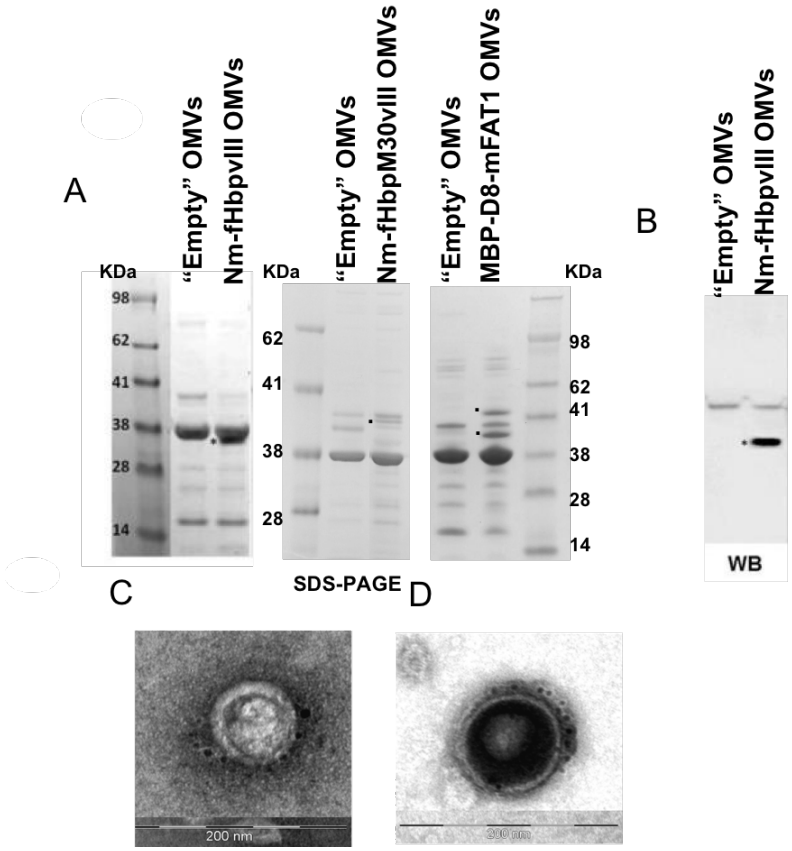


***Figure 8**. Expression of selected epitopes in OMVs. A) SDS-PAGE of OMVs. "Empty" OMVs, Nm-fHbpvIII OMVs, Nm-fHbpM30vIII OMVs and MBP-D8-mFAT1 OMVs were purified from BL21(DE3)ΔOmpA(pET21b+), BL21(DE3)ΔOmpA(pET-Nm-fHbp-vIII)), BL21(DE3)ΔOmpA(pET-Nm-fHbp-M30-vIII), and BL21(DE3) ΔOmpA(pET-MBP-D8-mFAT1), respectively. OMVs (20µg each) were loaded on SDS-polyacrylamide gels for SDS-PAGE analysis. Fusion proteins are indicated by the dots. B) WB analysis of "Empty" OMVs and Nm-fHbpvIII OMVs. After separation on SDS-polyacrylamide gel (1µg) proteins were transferred to nitrocellulose membrane and Nm-fHbpvIII was visualized using rabbit anti-EGFRvIIIpep antibodies. C-D) Immuno TEM analysis of Nm-fHbpvIII-OMVs purified using primary anti-EGFRvIIIpep rabbit antibodies and 5 nm gold-labeled anti-rabbit secondary antibody.*

3.3 Immunogenicity and protective activity of EGFRvIII-OMVs

Next, we analyzed the immunogenicity of Nm-fHbpvIII OMVs and their capacity to protect C57bl/6 mice from the challenge with B16F10EGFRvIII cell line. C57bl/6 mice (16 mice per group) were immunized with either "Empty" OMVs (control group) or with Nm-fHbpvIII OMVs. Vaccination was carried out by intraperitoneal (i.p.) injection at days 0, 14 and 28 (Figure 9A) and one week after the third immunization sera were collected and the induction of anti-

EGFRvIII-antibodies was confirmed by ELISA (Figure 9B). A good fraction of EGFRvIII-specific antibodies belonged to the IgG2a isotype, in line with previous data obtained in our laboratories showing that OMVs from E. coli BL21(DE3)*ΔompA* elicit a Th1-skewed immune response[89]. Next, at day 35, mice were challenged with an s.c. injection of 0.5 x 10^5 B16F10EGFRvIII cells and tumor growth was followed both in control mice and in mice immunized with Nm-fHbpvIII OMVs (Figure 9C). While all but one control mice developed large tumors 20 days after challenge (average tumor volume = 850 mm^3, with three mice sacrificed having developed tumors >1,500 mm^3), immunization with Nm-fHbpvIII OMVs markedly reduced tumor growth in a statistically significant manner. In particular, eight mice were completely protected while the remaining mice developed tumors with average volumes of approximately 400 mm^3.

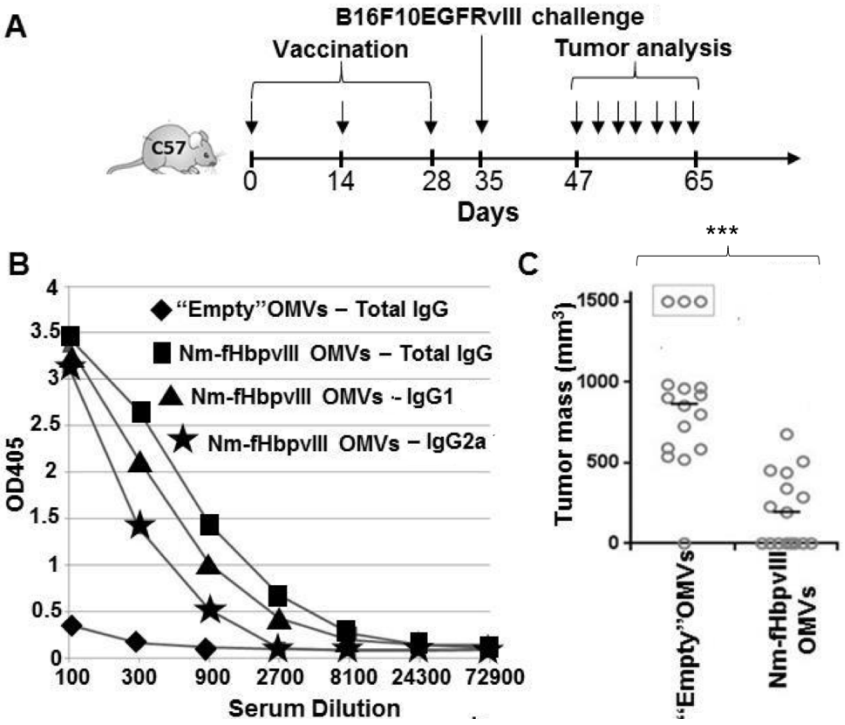


Figure 9. *Taken from manuscript under revision Grandi A. et al 2017. Immunogenicity and protective activity of Nm-fHbpvIII OMVs A) Schematic representation of immunization and challenge schedule in C57bl/6 mice. Mice (16 per group) were immunized three times (20μg/dose) with either "Empty" OMVs or with Nm-fHbpvIII OMVs. After 6 days after last dose, blood samples were collected to analyze anti-EGFRvIII antibody titers. The day after, mice were challenged s.c. with 0.5 x 10^5 B16F10EGFRvIII cells. Tumor development was followed over a period of 30 days after challenge. B) Anti-EGFRvIII antibody titers in C57bl/6 mice immunized with "Empty" OMVs and Nm-fHbpvIII OMVs. Sera from mice immunized as reported in A) were pooled and Total IgG, IgG1 and IgG2a were measured by ELISA, coating the plates with synthetic EGFRvIIIpep (0.5μg/well). C) Analysis of tumor development in C57bl/6 mice immunized with "Empty" OMVs and Nm-fHbpvIII OMVs and challenged s.c. with 0.5 x 10^5 B16F10EGFRvIII cells. The figure reports the tumor size in each mouse as measured at day 30. The three asterisks indicate a Pval≤0.001.*

3.4 Immunogenicity and protective activity of MBP-D8-mFAT1 OMVs

To confirm the ability of OMVs to induce anti-tumor immune responses in murine models, groups of BALB/c mice were immunized i.p. three times at day 0, 14 and 28 with 20 μg of MBP-D8-mFAT1 OMVs in Alum. After two weeks from the third immunization, sera were collected and pooled to analyze anti-mFAT1 antibodies. As shown in Figure 10A, good anti-mFAT1 antibodies were induced and such antibodies could bind to the surface of CT26 cell line as judged by flow citometry analysis (Figure 10B).

At day 35, mice were challenged with a s.c. injection of 1.5 x 10^5 CT26 cells and tumor growth was followed over a period of 30 days (Figure 10C). While all control mice developed large tumors 25 days after challenge, with three mice sacrificed having developed tumors >1,500 mm^3, immunized mice with MBP-D8-mFAT1 OMVs showed a markedly reduction with approximately 50% inhibition in tumor growth compared to the control group.

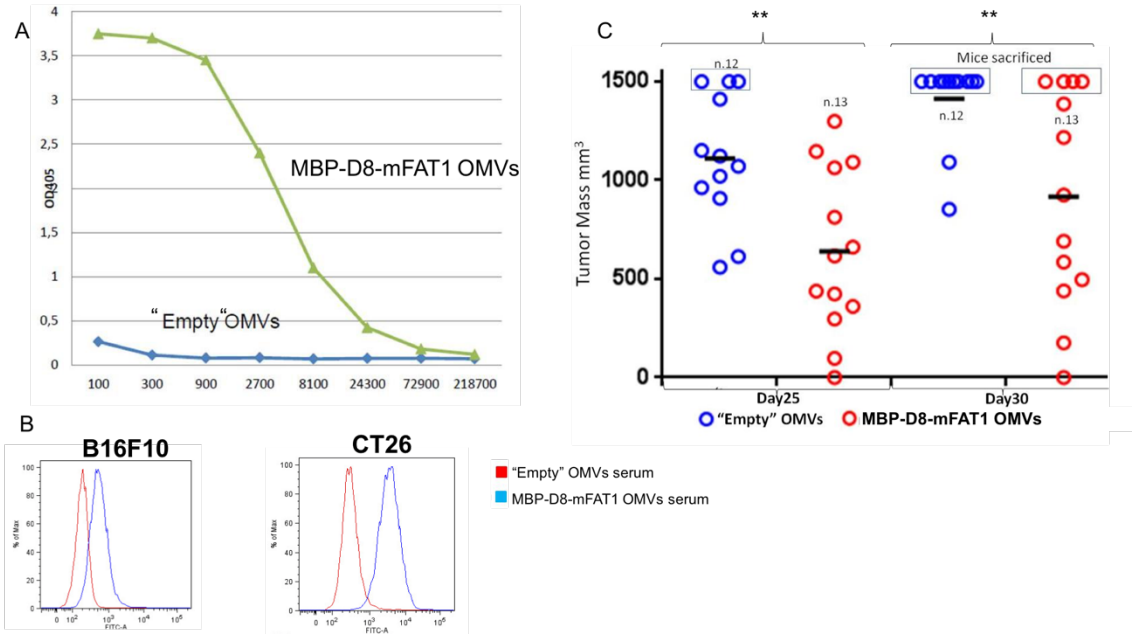


***Figure 10**. Immunogenicity and protective activity of MBP-D8-mFAT1 OMVs. A) Anti-mFAT1 antibody titers in BALB/C mice immunized with "Empty" OMVs and MBP-D8-mFAT1 OMVs. Sera from mice immunized were pooled and total IgG titers were measured by ELISA coating the plates with synthetic mFAT1 peptide (0.5μg/well). B) Flow citometry analysis on B16F10 and CT26 cell lines using sera collected from mice immunized with "Empty" OMVs and MBP-D8-mFAT1 OMVs. C) Analysis of tumor development in BALB/c mice immunized with "Empty" OMVs and MBP-D8-mFAT1 OMVs and challenged s.c. with 1.5 x 10^5 CT26 cells. The figure reports the tumor size in each mouse as measured at day 25 and 30 post cell challenge. The two asterisks indicate a Pval≤0.01.*

3.5 Synergistic protective activity of EGFRvIIIpep and M30

Having demonstrated that Nm-fHbpvIII OMVs induced a robust protection in C57bl/6 mice challenged with B16F10EGFRvIII cell line, we investigated whether protection could be further potentiated by using Nm-fHbpM30vIII OMVs expressing on their surface fHbp fused to three copies of CD4+ T cell M30 peptide followed by three copies of EGFRvIIIpep. Therefore, we set up an additional immunization/challenge experiment with three groups of mice. One group received "Empty" OMVs, a second group received Nm-fHbpvIII OMVs and the third group received Nm-fHbpM30vIII OMVs. Mice received three doses of vaccine two weeks apart and anti-EGFRvIIIpep antibodies were measured one week after the third immunization.

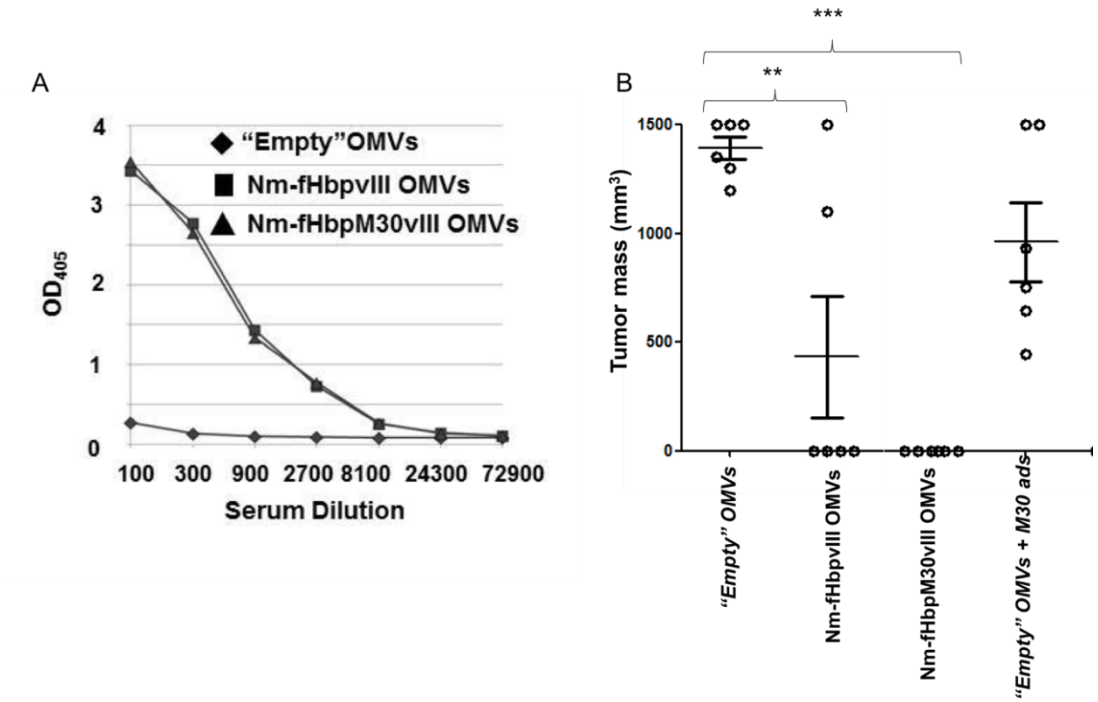


Figure 11. *Taken from manuscript under revision Grandi A. et al 2017. Immunogenicity and protective activity of Nm-fHbpvIII and Nm-fHbpM30vIII OMVs. A) C57bl/6 mice (6mice/group) were immunized three times with "Empty" OMVs, Nm-fHbpvIII OMVs, Nm-fHbpM30vIII OMVs (20µg/dose). Sera from immunized mice were pooled and Total IgG were measured by ELISA, coating the plates with synthetic EGFRvIIIpep (0.5µg/well). B) Analysis of tumor development in C57bl/6 mice immunized with "Empty" OMVs, Nm-fHbpvIII OMVs, Nm-fHbpM30vIII (20µg/dose) and "Empty" OMVs (20µg/dose) + M30pep (100µg/dose) and challenged s.c. with 0.5 x 10^5 B16F10EGFRvIII cells. The figure reports the tumor size in each mouse as measured at day 30 from challenge. The two asterisks indicate a Pval≤0.01 and the three asterisks indicate a Pval≤0.001.*

As shown in Figure 11A, Nm-fHbpvIII OMVs and Nm-fHbpM30vIII OMVs induced similar anti-EGFRvIII antibodies. Mice were then challenged with 0.5 x

10^5 B16F10EGFRvIII cells and tumor growth was followed over a period of 30 days. Figure 11 reports the result of this experiment. The robust protective activity of Nm-fHbpvIII OMVs was confirmed and protection was further potentiated in mice that received Nm-fHbpM30vIII OMVs. These mice were completely protected with no sign of tumor development at the site of injection. We have not tested yet the protective activity of OMVs engineered with M30 peptide alone but we tested the effect of immunization with synthetic M30 peptide "absorbed" to "Empty" OMVs and mice were only marginally protected by the vaccine (Figure 11B). Altogether these data provide a strong evidence that when engineered in OMVs, EGFRvIII and M30 epitopes work synergistically and together induce a robust immune response that results in complete protection against B16F10EGFRvIII cell line.

3.6 Synergistic protective activity of mFAT1 and CT26 exosomes

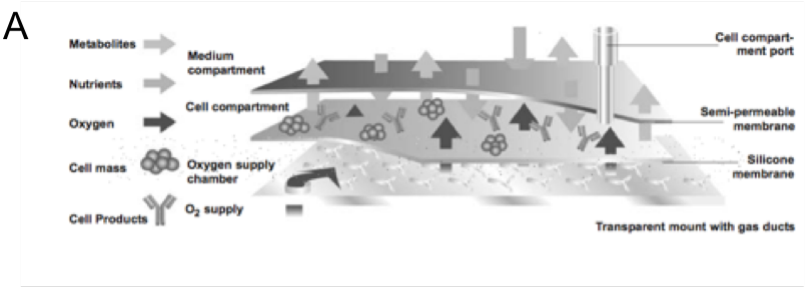
3.6.1 Purification of exosomes from cancer cell lines

Exosomes are usually prepared from standard cell culture flasks by differential ultracentrifugation, obtaining approximately 50-100 µg of exosomes from 200 ml of culture. Since this yield is not sufficient for immunization purposes, an important step of my experimental activity has been to identify a rapid effective way to get high and pure TEXs.

To this aim the integra CELLine culture system (schematically shown in Figure 12A from commercial protocol) was tested[109].

The CELLine bioreactor AD (for ADherent cells) is a two-compartment culture flask characterized by a cell compartment (15 ml maximum volume) and a medium compartment (1L maximum volume) separated by a 10 kDa semi-permeable membrane. Inoculated cells can adhere to a polyethylene terephtalate (PET) matrix of the cell compartment and exosomes are released in the medium which can be collected for vesicle recovery. High quality exosomes were recovered from cell supernatant and the yield was shown to be increased by at least fivefold (Figure 12B). Vesicle quality was assessed by WB using mAb against CD81 tetraspanin of six separated sequential preparations from CELLine bioreactor (Figure 12C).

A



B

	Flask 200ml	CELLine 15ml
Yield	50-100ug	300-500ug

C

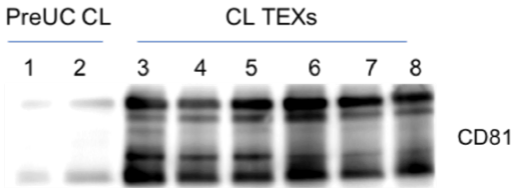


Figure 12. *(A) Schematic representation of CELLine bioreactor. The bioreactor is separated into a medium compartment and a cell compartment with a 10 kDa semi-permeable membrane. Cells inoculated can adhere to the PET matrix of the cell compartment and exosomes are released in the medium which are collected for vesicle purification. (B) Table reporting the TEXs yield isolating from culture flask conditioned medium or CELLine bioreactor supernatant following isolation and purification protocol as reported in Materials and Methods section, (C) Six TEXs preparations from CELLine bioreactor analyzed by WB for CD81 marker identification. Samples corresponding to pellets obtained from cell culture supernatants were obtained by centrifugation at 10,000 x g (PreUC CL- 20µg), followed by an ultracentrifugation step (CL TEXs). PreUC CL and CL TEX were visualized by WB analysis using mouse anti-CD81 antibodies.*

Naturally released TEXs were therefore isolated and purified from cell culture supernatants of human HCT15 or mouse CT26 colorectal or mouse B16F10 melanoma cancer cell lines. TEX quality was assessed by verifying the presence of known exosome markers using WB analysis and by TEM using negative staining and immune gold particles (Figure 13). In particular, total cell extracts (CELL - 20µg) and TEXs (5µg) from HCT15 cell line were loaded on SDS-PAGE and analyzed by WB (Figure 13A) with antibodies specific for CD81 and for the lipid raft associated protein Flotilin-1. TEXs samples were negative for Calnexin, an endoplasmic reticulum marker, indicating that the exosome samples contained little contamination from cell debris.

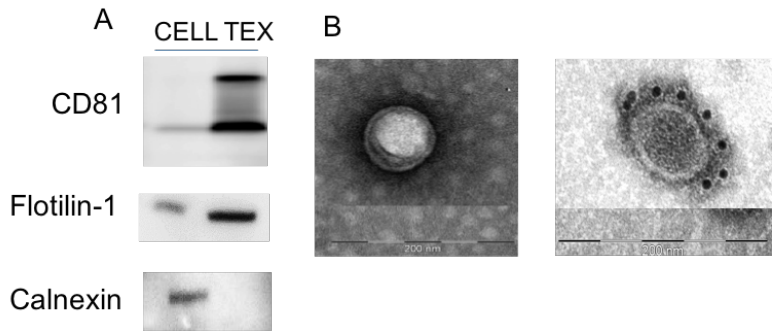


Figure 13. *TEXs quality control. (A) TEXs were isolated from HCT15 cell line and samples corresponding to total cell extracts from HCT15 cell line (CELL- 20µg) and TEXs isolated from HCT15 cells (TEX - 5µg) were analyzed by WB for CD81 and Flotilin-1 marker identification. Calnexin was used as negative control thus revealed in cell extracts, CELL, but not in TEX samples. B) TEM analysis of TEXs purified using negative stain (left) and with primary anti-CD81 mouse antibodies and 10 nm gold-labeled anti-mouse secondary antibody (right).*

3.6.2 Exosome-OMV interaction

Next we investigated whether TEXs could spontaneously interact with OMVs and generate complexes which would enable the co-delivery of exosome-associated tumor antigens and OMV-associated immunostimulatory molecules to APCs. Co-delivery of antigens and adjuvants to the same APCs is in fact a prerequisite to induce an optimal adaptive immune response. The rationale for testing the spontaneous interaction between the two vesicles stem from the notion that OMVs actively interact with eukaryotic cell membrane and there are reports which suggest that such interaction lead to the OMV-membrane fusion. If such fusion does occur, the two vesicles should theoretically be capable of fusing together.

To analyze TEXs/OMVs interaction we first set-up methods to visualize them and discriminate them when mixed together. Two methodologies were used: confocal microscopy and TEM.

For confocal microscopy, TEXs purified from the murine melanoma B16F10 cell line, were fluorescently labeled (green) by incubation with Exo-Glow dye (SBI, Mountain View, CA). Exo-Green stain is based on Carboxyfluorescein succinimidyl diacetate ester (CFSE) chemistry (Figure 14A), a compound which is membrane permeable. When CFSE enters the vesicles, it is hydrolyzed by endogenous esterases, removing the diacetate residues. This generates a fluorescent green product, which interact with NH_2 groups and make TEXs fluorescent. When fluorescent TEXs (Exo-green TEXs) were incubated with

B16F10 cells, they interacted with the cell membranes and after 2 hour incubation at 37°C were found in the intracellular compartment (Figure 14B).

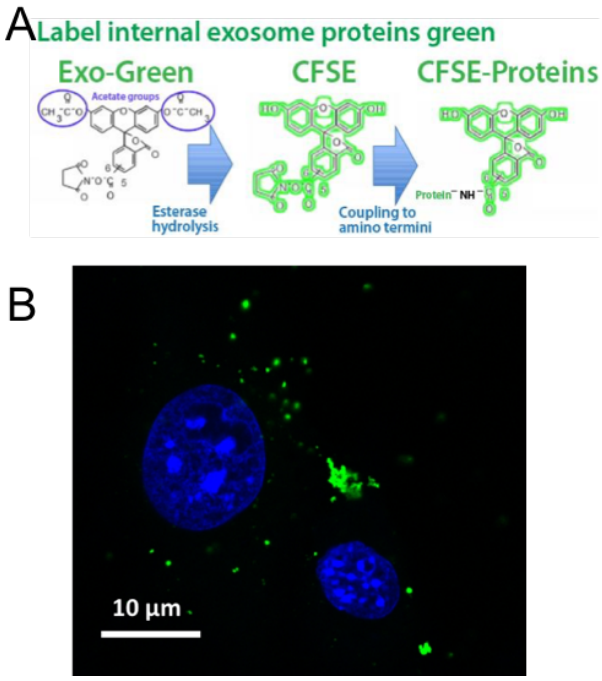


Figure 14. *TEXs fluorescently labeled with Exo-Green. A) Schematic presentation of Exo-green dye. Based on CFSE chemistry this compound is membrane permeable and once entered the vesicles, it is hydrolyzed by endogenous esterases which generates a fluoresce green product, that make TEXs fluoresce green (Exo-green TEXs). B) Exo-green TEXs were incubated with B16F10 cell, and uptake was visualized by confocal microscopy. Nuclei were stained in blue using DAPI (4',6-diamidino-2-phenylindole).*

In parallel, OMVs were made red fluorescent by expressing the fluorescent protein mCherry fused to the periplasmic MBP protein. mCherry is a monomeric protein with peak absorption/emission at 587 nm and 610 nm, respectively[14]. The fusion protein was expressed in *E. coli* BL21(DE3)*ΔompA* strain and OMVs were isolated and purified. As shown and highlighted in Figure 15A, mCherry efficiently compartmentalized in OMVs.

When fluorescent OMVs (OMV-red) were incubated with B16F10 cells, they rapidly interacted with the cell membrane and after 2 hour incubation at 37°C a fraction of OMVs were found in the intracellular compartment, indicating that they were rapidly endocytosed (Figure 15B).

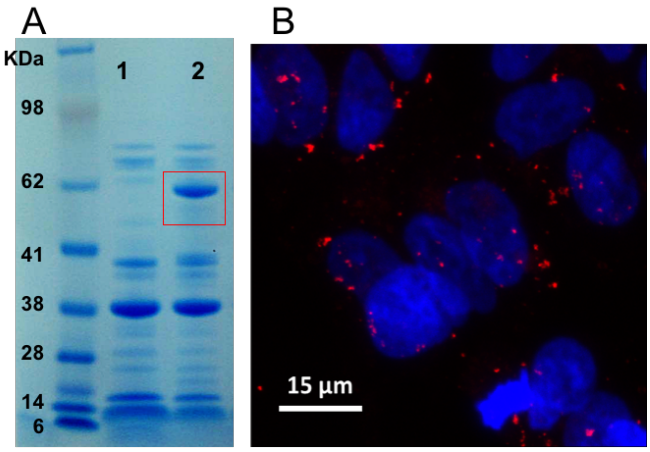


Figure 15. *Expression of mCherry fluorescent protein in OMVs. A) SDS-PAGE of OMVs. OMVs were purified from BL21(DE3)ΔOmpA(pET21b+), ("Empty" OMVs) and BL21(DE3)ΔOmpA(pET-MBP-mCherry) (OMV-red) and loaded on SDS-polyacrylamide gel for SDS-PAGE analysis (20µg OMVs). mCherry is shown to compartmentalize in OMVs (lane 2). B) OMV-red were incubated with B16F10 cells and uptake was visualized by confocal microscopy. Nuclei were stained in blue using DAPI.*

Having set-up the methods to fluorescently label the two vesicles in a way that they could be discriminated, their possible interaction was followed by mixing them in a diluted solution. Under this condition, the visualization of sufficient co-localization events can be attributed to relatively stable interactions between the two particles rather than random collisions which can take place if relatively concentrated solutions were used.

As shown in Figure 16A, Exo-green TEXs and OMV-red fluorescent signals appeared to co-localize with relatively high frequencies, suggesting that when the two vesicles interact they form relatively stable complexes.

To further confirm TEX-OMV interaction, the two fluorescence-labelled vesicles were first allowed to interact at 4°C for an overnight (o/n) and then incubated 2 hours with the murine melanoma B16F10 cell line. As shown in Figure 16B, fluorescent signals of both vesicles could be visualized within the same cell. Even if this experiment does not directly demonstrate the interaction between TEXs and OMVs, it provides an important evidence that, regardless their physical status, the two vesicles can potentially be co-delivered to APCs *in vivo*.

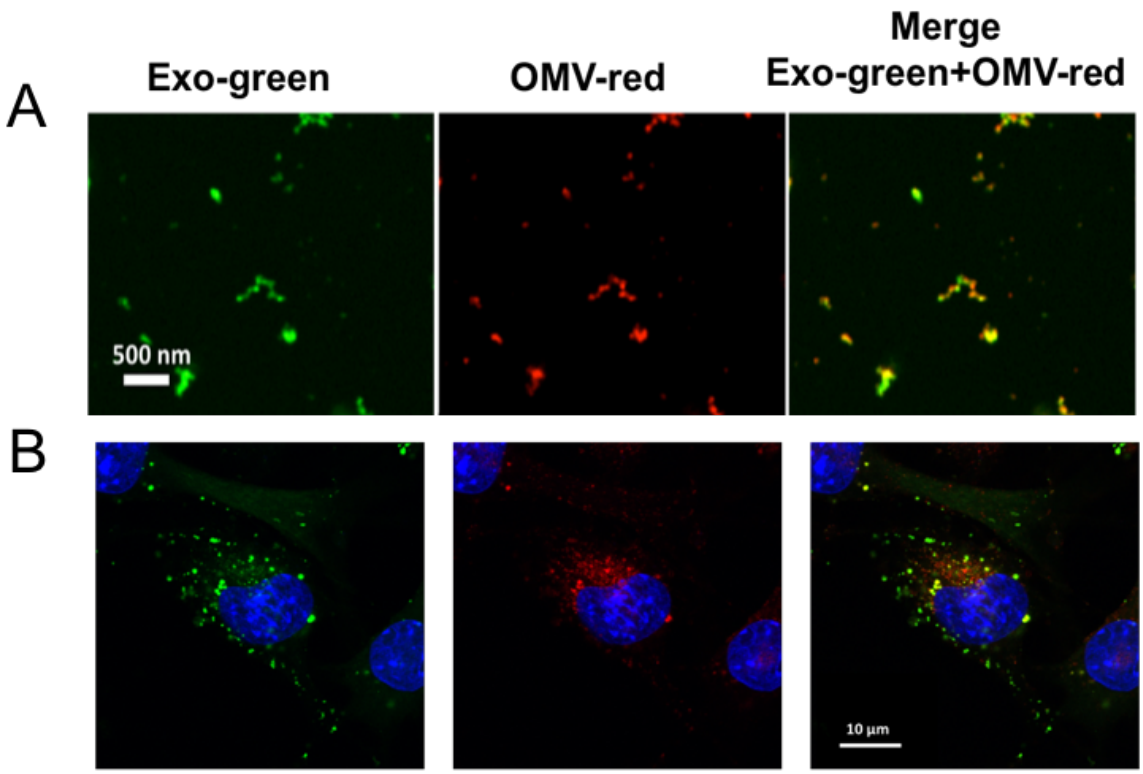


Figure 16. *TEX-OMV interaction and cell uptake. A) Exo-green TEXs and OMV-red were incubated in a 1:1 ratio at 4°C o/n and fluorescent signals were visualized by confocal microscopy. B) Exo-green TEXs and OMV-red were incubated at 4°C o/n in a 1:1 ratio and subsequently with B16F10 cells for 2 hours at 37°C. Fluorescent signals of the vesicles are visualized within the same cell. Nuclei were stained in blue using DAPI.*

OMVs-exosomes colocalization/interaction was also analyzed by immune stained TEM. For this experiment, TEXs were purified from the human colorectal cancer cell line HCT15 while OMVs were purified from *E. coli* BL21(DE3)*ΔompA*(pET-Nm-fHbp-vIII) producing vesicles decorated with EGFRvIIIpep epitope. After incubation with mouse anti-CD81 antibodies, TEXs could be visualized using anti-mouse antibodies labelled with 10 nm gold particles. By contrast, OMVs could be visualized by incubation with rabbit anti-EGFRvIIIpep antibodies and subsequent incubation with anti-rabbit antibodies labelled with 5 nm gold particles.

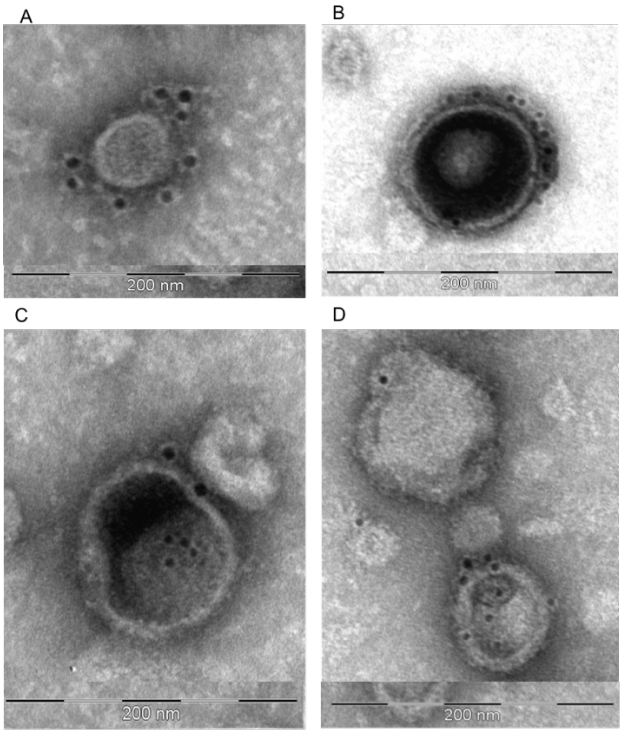


***Figure 17**. Immuno TEM analysis of TEXs and OMVs interaction. A) TEXs purified from HCT15 cell line were incubated with primary anti-CD81 mouse antibodies and 10 nm gold-labeled anti-mouse secondary antibody B) Nm-fHbpvIII OMVs incubated with primary anti-EGFRvIIIpep rabbit antibodies and 5 nm gold-labeled anti-rabbit secondary antibody. C-D) TEXs and OMVs were incubated together at 4°C o/n and interaction events were visualized by Immuno TEM.*

As shown in Figure 17 A and B, both TEXs and OMVs could be stained with their respective antibodies. When the two vesicles were mixed together (Figure 17 C and D) several TEX-OMV interaction events could be detected. Such interactions did not appear to involve membrane fusions but rather physical contact between the two membranes. Discrimination between OMVs and TEXs was possible first on the basis of their respective size (OMVs showed an average size of 100 nm as opposed to the 50 nm average size of TEXs) and second on the basis of the associated gold particles. All TEXs were associated to 10 nm gold particles while OMVs to 5 nm gold particles.

3.6.3 Assessment of immunogenicity and protective activity of TEXs-OMVs complexes

In the previous section, we have reported the experimental evidence that OMVs and TEXs can spontaneously interact. The interaction appears to generate complexes that potentially can co-deliver and can be phagocytosed by the same APC, a feature necessary for the elicitation of an effective immune

response against TEXs proteins. We therefore, analyzed whether the combination of MBP-D8-mFAT1 OMVs and TEXs from CT26 cell line could exert a possible additive or synergistic protective effect in syngeneic challenged mice using CT26 cell line.

BALB/c mice were immunized four times, one week apart, with either MBP-D8-mFAT1 OMVs in Alum or with the combination of MBP-D8-mFAT1 OMVs and TEXs from CT26 cells in Alum mixed in a 1:1 ratio. A week after the last immunization sera were collected and anti-TEXs IgG, IgG1 and IgG2a were measured by ELISA, coating the plates with 5 µg/ml of TEXs/well. As shown in Figure 18 B-D, TEXs-OMVs immunization elicited anti-TEXs antibodies and, as expected, the immune response was skewed toward a Th1-type of response as indicated by the presence of a good level of anti-TEXs IgG2a antibodies. The day after sera collection, mice were challenged s.c. with 1.5 x 10^5 CT26 cells, and tumor growth was followed over a period of 21 days by measuring tumor size with a caliper. As shown in Figure 18E, at day 21 mice treated with MBP-D8-mFAT1 OMVs developed tumors with an average size of 500mm^3, a number statistically different from the average tumor size of control mice immunized with “Empty” OMVs (Pval<0.05). Interestingly, tumor growth inhibition was further increased when mice were immunized with the MBP-D8-mFAT1 OMVs-TEXs mixture (average tumor size: 380mm^3; Pval<0.001). Although confirmation using a larger group of mice is needed, these data provide evidence that both D8-mFAT1 and exosome-associated cancer antigens contribute to inducing, in the presence of OMVs, an anti-CT26 immune response.

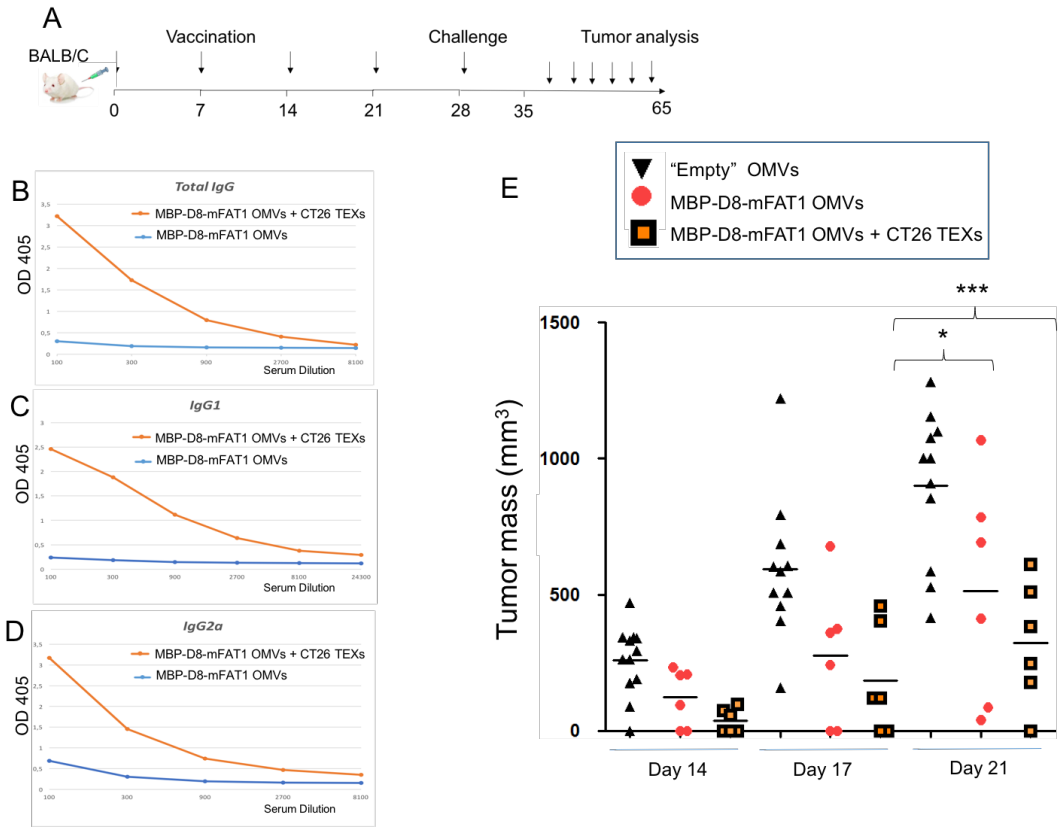


***Figure 18**. A) Schematic representation of immunization and challenge schedule in BALB/c mice. Mice were immunized four times with either "Empty" OMVs (20µg/dose), with MBP-D8-mFAT1 OMVs (20µg/dose) or with MBP-D8-mFAT1 OMVs + TEXs isolated from CT26 (CT26 TEXs) (20µg/dose, 1:1 ratio) in Alum. After 6 days from last dose, blood samples were collected to analyze antibody titers. The day after, mice were challenged s.c. with 1.5 x 10^5CT26 cells. B-C-D) Anti-TEXs antibody titers in BALB/C mice immunized with "Empty" OMVs (20µg/dose), with MBP-D8-mFAT1 OMVs (20µg/dose) and with MBP-D8-mFAT1 OMVs + CT26 TEXs (20µg/dose, 1:1 ratio) in Alum. Sera from mice immunized as reported in A) were pooled and Total IgG, IgG1 and IgG2a were measured by ELISA, coating the plates with TEXs from CT26 (5µg/ml). E) Analysis of tumor development in BALB/C mice immunized with OMVs and challenged s.c. with 1.5 x 10^5CT26 cells. The figure reports the tumor size in each mouse as measured at day 21. One asterisk indicates a Pval<0.05 and the three asterisks indicate a Pval<0.001.*

4 DISCUSSION

This PhD Thesis describes the experimental activities I have directly contributed to either as principal investigator or as collaborator, addressing the question of whether bacterial OMVs can be exploited as a platform for cancer immunotherapy.

OMVs are being extensively and successfully utilized in the preclinical and clinical settings for prophylactic vaccination against infectious diseases. Their unique adjuvanticity, which directs the immune responses toward a marked Th1 profile, and the ease with which they can be manipulated and purified have attracted the attention of several academic and industrial groups and bacterial OMV-based vaccines are already available for human use. However, information regarding the applicability of this platform technology in cancer vaccines is still limited.

We clearly show that OMVs engineered with EGFRvIII, a tumor-specific B cell epitope expressed in several human tumors, induce high anti-EGFRvIII antibody titers with a Th1 skewed response. Furthermore, anti-EGFRvIII antibodies were capable of reducing the growth of EGFRvIII-positive B16F10 cancer cells in a syngeneic immune competent mouse model. Protection levels appeared to be similar to those described by Heimberger and co-workers[106] using the same mouse model and a KLH-conjugated EGFRvIII peptide in the presence of GM-CSF.

The ability of OMVs to induce protective antibody-mediated immune responses was further supported by expressing D8-mFAT1, a second tumor-associated B cell epitope recently discovered in our laboratories[39]. FAT1 is a tumor antigen up-regulated in most human colon cancers. A monoclonal antibody binding to a specific domain of FAT1 (D8) inhibits tumor growth in xenograft mouse models. Since we found that FAT1 mouse homolog is also over-expressed in CT26 mouse colon cancer cell line, we decorated OMVs with the corresponding D8 mouse epitope and we demonstrated that the engineered vesicles induced anti-D8-mFAT1 antibodies, which protected syngeneic BALB/c mice upon tumor challenge using CT26 cancer cells.

A second important message from the work we performed is that by decorating OMVs with more than one antigen (two antigens in the work reported in this PhD Thesis) the antigens appear to work synergistically, potentiating the overall protective efficacy of the vaccine formulation. We combined the EGFRvIII B cell epitope with a tumor-specific CD4+ T cell epitope (M30) recently reported to be expressed in B16F10 cell line as a consequence of spontaneous mutation. OMVs engineered with both antigens elicited anti-EGFRvIII and anti-M30 immune responses in immunized C57bl/6 mice and responses completely protected mice from the challenge with EGFRvIIIB16F10 cell line.

This is an interesting observation in light of the fact that in glioblastoma patients, vaccination with EGFRvIII-conjugated peptide was shown to prolong overall survival but ultimately EGFRvIII-negative tumor cells escape vaccine-induced protection. This immunoediting mechanism in part explains the failure of *Rindopepimut* (a vaccine based on EGFRvIII peptide) for glioblastoma treatment in a phase III trial. Our data pointing to the synergistic effect of EGFRvIII-OMV in combination with other cancer-specific epitopes might rejuvenate the interest in EGFRvIII antigen in the near future.

Considering the ease with which OMVs can be manipulated with foreign antigens, these results lead to the attractive possibility of exploiting the OMV platform in cancer precision medicine. Expression of multiple neo-epitopes, in single or repeated copies, on the same or in separated OMVs are all potential strategies which are going to be deepened in our nearest future work.

In light of our promising data on the beneficial effect of the multiple-antigen OMV decoration strategy, an important part of my activity was dedicated to analyse whether the combination of cancer-derived exosomes (TEXs) with OMVs could represent a valid strategy for cancer immunotherapy. The rationale of this approach stems from two experimental observations. First, cancer cells abundantly release TEXs and these have been shown to incorporate several tumor-specific and tumor-associated antigens. Therefore, exosome represent a natural source of tumor antigens. Second, OMVs actively interact with eukaryotic cell membrane and there are reports which suggest that such interaction lead to OMV-cell membrane fusion. If such fusion between OMV

membrane and cell membrane does occur, it could be possible that not only exosomes and OMVs can interact with each other but also the two vesicles could theoretically be capable of creating fusions. The existence of a physical interaction between exosomes and OMVs would have an important immunological implication since it would enable the co-delivery of exosome-associated tumor antigens and OMV-associated immunostimulatory molecules to APCs. Co-delivery of antigens and adjuvants to the same APCs is a prerequisite to induce an optimal adaptive immune response.

The physical association of TEXs with OMVs was analysed by confocal microscopy and then by TEM. Such analyses showed that when mixed together at least a fraction of the two vesicle populations did associate. Such association did not appear to lead to membrane fusion, even though a few TEM images seem to provide examples of “intimate” interactions (Figure 17).

Regardless the nature of TEX-OMV interaction, the combination of the two vesicles appeared to elicit anti-tumor immune responses. This was demonstrated by combining MBP-D8-mFAT1 decorated OMVs with CT26-derived TEXs. Immunization of BALB/c mice with MBP-D8-mFAT1 OMVs partially protected BALB/c mice from the challenge of CT26, a cell line that expresses mFAT1 on its surface. In addition, the immunization with MBP-D8-mFAT1-OMVs + TEXs derived from CT26 further improved the anti-CT26 protective activity. The treatment appeared to be safe in mice with no signs of toxicity, in line with the reported human safety data of a vaccine formulated with GM-CSF and TEXs isolated from ascites fluid of colorectal cancer patients[70].

Considering the relative abundancy of cancer antigens in TEXs, although promising these results suggest that additional work is needed to fully exploit the potential of exosomes in cancer immunotherapy. There are several aspects that require further investigation.

First, as pointed out previously, the TEX-OMV interaction is only partial under the experimental conditions used. It would be interesting to see whether the addition of lipophilic components known to promote membrane fusion can increase and strengthen the vesicle association. In this respect, the establishment of a stable association might be crucial to prevent the physical

dissociation of the two vesicles at the site of injection.

Second, the ratio used to promote TEX-OMV association might not be the optimal one. In these experiments only 1:1 ratio (protein content) was tested but other conditions might favor more proficient interactions.

Third, the route of immunization might also have a role in the quality and quantity of immune response. In the recent work by Kranz and co-workers[110], it has been demonstrated that negatively charged liposomes carrying TAA-encoding synthetic RNAs were efficiently taken up by splenic DCs when administered i.v. This resulted in a potent elicitation of TAA-specific CD4+ and CD8+ T cells. Since OMVs are known to be negatively charged, i.v. injection might promote the efficient delivery of TEX-OMV complexes to the spleen.

Finally, it is known that TEXs carry components that can potentially inhibit the immune response, such as the transforming growth factor-ß (TGF-ß) and CD47, which provides the “don’t eat me” signal[111]. Since the inhibitory activity of CD47 can be potentially blocked by anti-CD47 antibodies, it would be interesting to investigate further whether CT26-derived exosomes carry CD47 and, should this be the case, whether the co-administration of anti-CD47 antibodies can potentiate the immune responses of TEX-OMV vaccination.

5 MATERIALS AND METHODS

5.1 Chemicals, cell lines and animals

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, sodium pyruvate (SP), MEM non- essential amino acids (NEAA) and Fetal Bovine Serum (FBS) were purchased from Gibco-Life Technologies. Penicillin/streptomycin/L-glutamine (PSG) was purchased from Euroclone.

Human colorectal adenocarcinoma HCT-15 cell line, was obtained from ATCC (Manassas, VA, U.S.) and cultured under recommended conditions. Mouse melanoma B16F10 and mouse carcinoma cells derived from colon CT26 cell lines, were kindly given by the Department of Biomedical and Clinic Sciences of the University of Florence. Melanoma B16F10 cell line stably expressing EGFRvIII was kindly provided by Prof. J. H. Sampson from the Department of Neurosurgery of the Duke University Medical Center in North Carolina (U.S.). Stock preparations of cells were stored at -80°C and/or liquid nitrogen in 90% FBS and 10% dimethyl sulfoxide (DMSO). Cells were tested for mycoplasma before animal injection.

C57bl/6 and BALB/c female 4 weeks old mice were purchased from Charles River Laboratories and kept and treated in accordance with the Italian policies on animal research at the Toscana Life Sciences animal facility (Siena, IT).

5.2 Bacterial strains and culture conditions

Plasmid assembly using the polymerase incomplete primer extension (PIPE) method[112] was carried out in *E. coli* HK-100 strain. OMVs were purified from *E. coli* BL21(DE3)*ΔompA* strain[89]. *E. coli* were routinely grown in Luria-Bertani (LB) broth medium (Difco) at 37°C and when required, Ampicillin (Amp) was added to a final concentration of 100 µg/ml. Stock preparations of strains in LB and 15% glycerol were stored at -80°C. Each bacterial manipulation was started from an o/n culture inoculum of a frozen stock or of a single colony from LB plate.

5.3 Construction of plasmids

Plasmid assembly using the polymerase incomplete primer extension (PIPE) cloning method[112] was applied for plasmid construction.

pET21-Nm-fHbp and the pET-Nm-fHbp-vIII plasmids expressing the *Nm*-fHbp and *Nm*-fHbp fused to three copies of EGFRvIIIpep, respectively were generated as previously described from Fantappiè et al 2017[92]. pET21-MBP plasmid was generated as previously described from Fantappiè et al 2014[89]. pET-Nm-fHbp-M30-vIII plasmid carries the *Nm*-fHbp gene fused to a synthetic DNA fragment encoding three copies of B16-M30pep and three copies of EGFRvIIIpep, each copy intercalated by a Glycine-Serine spacer. pET-Nm-fHbp-M30-vIII plasmid was generated as described in paper under revision Grandi A. et al 2017.

To clone the mCherry protein as translational fusion gene to the C-terminus of MBP, a DNA fragment named mCherry was amplified by PCR using mCh3FW/mCh3Rev primers from the plasmid pcDNA3-MACA-HXB2-mCherry WPRE (kindly given by the laboratory group of Prof. Cereseto in the University of Trento). A spacer containing the Gly-Gly-Ser aminoacids was introduced between the MBP and the mCherry proteins in order to obtain a sufficient linker flexibility and reduced negative folding interference[113]. pET21-MBP plasmid was amplified using nohisflag/mCh2Rev primers (Table II) to generate a linear plasmid. Finally, the PCR products were mixed together and used to transform *E. coli* HK100 competent cells, thus obtaining pET-MBP-mCherry plasmid.

To clone D8-mFAT1 peptide as translational fusion to the C-terminus of MBP a DNA fragment, named D8-mFAT1x3, coding for three copies of D8-mFAT1 (IQVEATDKDLGPSGHVTYAILTDTE), was assembled via PCR annealing steps. Plasmid pET21-MBP has been used as template for a PCR reaction carried out according to the PIPE method to generate a linear plasmid, using primers pET21-MBPF and pET21-MBPR (Table II).

D8-mFAT1x3 was assembled *in vitro* using the six synthetic oligonucleotides reported in Table II and subsequently amplified by PCR with primers MBPmFA-F and MBPmFA-R. Finally, D8-mFAT1x3 fragment and linearized plasmid were

mixed together and transformed in *E. coli* HK100 competent cells, obtaining pET-MBP-D8-mFAT1 plasmid.

Nohisflag	taacatcaccatcaccatcacgattacaaaga
mCh3FW	accaagggcggtagcatggtgagcaagggtgag
mCh3Rev	gtgatggtgatgttacttgtacagctcgtccatgc
mCh2rev	caccatgctaccgcccttggtgatacgagtctgcg
mFa-F1	atccaagtggaggcgaccgataaagacctgggtccgtcggggcatgtg
mFa-R1	aacctgaatttcggtgtcggtcaggatggcatacgtcacatgccccgacgg
mFa-F2	accgaaattcaggttgaagccaccgacaaagacttaggcccgagtggtcac
mFa-R2	ctgaatttcagtatcggtgagaatcgcgtaggtcacgtgaccactcgggcc
mFa-F3	gatactgaaattcaggttgaagctaccgataaagatttgggcccgagtggt
mFa-R3	ttcagtatccgtgaggatcgcataggttacatgaccactcgggcccaa
MBPmFA-F	cgcgcagactcgtatcaccaagatccaagtggaggcg
MBPmFA-R	tcgtgatggtgatggtgatgttattcagtatccgtgag
pET21-MBPF	catcaccatcaccatcacgattac
pET21-MBPR	cttggtgatacgagtctgcgcgtc

Table II. *Primer's sequences list*

5.4 Expression of the heterologous proteins in E. coli BL21(DE3)ΔOmpA strain and OMVs preparation

Plasmids containing the genes of interest were used to transform *E. coli* BL21(DE3)*ΔompA* strain. Recombinant clones were grown in LB medium (starting OD_{600} = 0.05) and, when the cultures reached an OD_{600} value of 0.5, protein expression was induced by addition of 1 mM isopropyl-ß-D-galactopyranoside (IPTG, Sigma-Aldrich). After 2 hours, cultures were precleared from living bacterial cells by a centrifugation step at 6,000 x g for 30 minutes followed by a filtration through a 0.22 µm pore size filter (Millipore). Supernatants were then subjected to high-speed centrifugation (200,000 x g for 2 hours) and pellets containing the OMVs were finally re-suspended in sterile 1X PBS. OMVs amounts were estimated by measuring protein concentration using DC protein assay (Bio-Rad). Total bacterial lysates were prepared by suspending bacterial cells from a volume culture corresponding to OD_{600} = 1 (centrifuged at 13,000 X g for 5 minutes) in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) Laemli buffer (Bio-Rad) and heated at 100°C for 5 minutes. Similarly, 30µg of OMVs were prepared in SDS-

PAGE loading buffer. Each sample (10µl final volume) was separated by 4-12% SDS-PAGE gel (Thermo Fisher Scientific), run in MES or in MOPS buffer (Thermo Fisher Scientific) and stained with Coomassie Blue.

5.5 Exosome isolation and purification

Cells were cultured with complete medium (DMEM or RPMI) composed by PSG (1X), SP and NEAA supplemented (1mM), with 10% exosome-depleted FBS (depleted of serum exosomes by o/n high speed centrifugation at 110,000 x g) in T175 flasks for 48 hours or in CELLine AD 1,000 bioreactor (INTEGRA). TEXs were purified from harvested cell supernatant by subsequently differential centrifugation steps. Centrifugations with increasing speed were performed and intended to pellet consecutively cells (300 x g, 10 minutes), apoptotic bodies (2,000 x g, 15 minutes) and shedding vesicle/microparticles/ectosomes (10,000 x g, 30 minutes), followed by filtration through a 0.22 µm pore size filter (Millipore). A final high speed centrifugation step at 110,000 x g for 114 minutes and a washing step with sterile 1X PBS could pellet exosomes, finally suspended in sterile 1X PBS. TEXs amounts were estimated by measuring protein concentration using the DC protein assay (Bio-Rad).

5.6 CELLine bioreactor

The two-compartment bioreactor CELLine (INTEGRA) is composed by a medium compartment, which contains up to 1L medium, and a cell compartment containing cells in 15ml medium. These compartments are individually accessible, but separated by 10kDa semi-permeable cellulose acetate membrane.

To start $25x10^6$ cells were plated in 15ml growth medium at day 0. The first harvest was made 7 days after inoculation and submitted to purification protocol as described in section *5.5 Exosome isolation and purification*. Cells were split 1:10 and inoculated in the cell compartment. Subsequent harvesting and purification of supernatant procedures were performed every 4-7 day cycles, depending on the cell type used, the viability and individual growth characteristics. Cellular viability was routinely checked by collecting 1ml supernatant from cell compartment and was shown to be always higher or

equal to 90%. Cellular viability was assessed using Trypan Blue Solution 0,4% (Thermo Fisher Scientific) in Countess™ Cell Counter (Life Technologies).

5.7 Western blot analysis

Purified OMVs or TEXs (1µg or 5µg protein content) were suspended in SDS-PAGE loading buffer (Bio-Rad) and were then separated on a SDS-PAGE 4-12% polyacrylamide gel (Thermo Fisher Scientific). Proteins separated by SDS-PAGE were subsequently transferred onto nitrocellulose membrane by standard methods. The filters were blocked at room temperature (RT) for 1 hour by agitation in blocking solution (10% skimmed milk and 0.05% Tween 20 dissolved in PBS (TPBS)), followed by o/n incubation at 4°C with the required monoclonal or polyclonal antibody (mAb or pAb). The pAbs against EGFRvIIIpep (Genscript) was used at 1µg/ml concentration in 1% skimmed milk-TPBS. mAb against CD81 tetraspanin (BD Pharmigen™) or against Flotilin-1 or Calnexin (BD Biosciences) were used at 2 µg/ml in 1% skimmed milk-TPBS. After three washing steps in TPBS, the filters were incubated in 1:2,000 dilution of peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Dako) for 1 hour in 1% skimmed milk-TPBS, and after 3 washing steps in TPBS, antibody binding was detected by using the SuperSignal West Pico chemiluminescent substrate (Pierce) at Image Quant LAS4,000 (GE Heathcare) instrument.

5.8 TEXs labeling with Exo-green

TEXs were labelled with Exo-green label (SBI- Exo-Glow) following suggested product's protocol. Briefly, 10X Exo-Green dye was ten-fold diluted in 500µl 1X sterile PBS containing TEXs (100µg) and incubated at 37°C for 10 minutes. Labelling reaction was stopped adding 100µl ExoQuick –TC reagent to the sample, then placed in ice for 30 minutes. Samples were centrifuged for 3 minutes at 20,000 x g. TEXs pellet was suspended in 500µl 1X sterile PBS and analysed by confocal microscopy.

5.9 Confocal microscopy for TEXs-OMVs analysis

Firstly, cells (1.5 x 10^5/well) were plated on microscope coverslips in 6 multi-

wells plate and let adhere at 37°C o/n.

Subsequently, TEXs labelled with Exo-green (Exo-green TEXs) and OMVs expressing mCherry fluorescent protein in their lumen (OMV-red), were incubated in a protein ratio 1:1, at 4°C o/n in 1ml 1X sterile PBS final volume. Exo-green or OMV-red were used alone as controls. After o/n incubation, vesicles were incubated with cells at 37°C for 2 hours and washed twice with 1X sterile PBS. Nuclei were stained blue using DAPI (300nM). Internalisation and co-localization of the fluorescent signals were assessed by a laser-scanning confocal microscope with 488nm/594nm laser lines.

Alternatively, after o/n incubation at 4°C, interacting Exo-green and OMV-red were directly visualised at the laser-scanning confocal microscope with 488nm/594nm laser lines.

5.10 TEM immunogold

Before immune Transmission Electron Microscopy (TEM), cross-reactivity between the antibodies was excluded by WB analysis (see section *5.7 Western blot analysis* for details) and, as shown in Figure 19: “Empty” OMVs (lane 1), Nm-fhbpvIII OMVs (lane 2), TEXs isolated from HCT15 (lanes 3-4) were analyzed using rabbit anti-EGFRvIII pAb (Figure 19A) and mouse anti-CD81 mAb (Figure 19B) revealed with peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin.

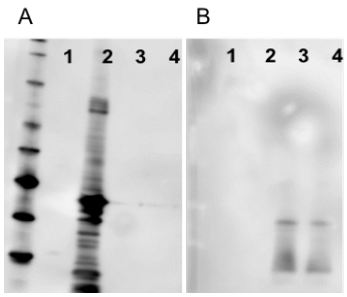


***Figure 19**. WB analysis for antibody cross reactivity control: “Empty” OMVs (lane 1), Nm-fhbpvIII OMVs (lane 2), TEXs isolated from HCT15 cell line (lanes 3-4) were analyzed using anti-EGFRvIII pAb (A) and mAb anti-CD81 (B) and revealed with peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin.*

Once excluded cross-reactivity between the antibodies, purified OMVs and/or TEXs, were visualized using TEM. Briefly, a 5 µl aliquot of purified vesicles preparation with a final concentration of 20 ng/µl was applied to 200-square

mesh nickel grids coated with a thin carbon film (Agar Scientific) and let stand for 3 minutes at RT. The samples were then blocked in 0.5% BSA dissolved in PBS for 1 hour at RT. Subsequently the samples were incubated with primary rabbit anti-EGFRvIII pAb (OMVs) and/or with mouse anti-CD81 mAb (TEXs) for 1 hour at RT. Grids were washed 3 times in blocking buffer and incubated with 5-nm gold-labeled anti-rabbit secondary antibody and/or with 10-nm gold-labeled anti-mouse secondary antibody (BB International (Madison, WI)) for 1 hour at RT. Immunostained vesicles were then negatively stained in 1% phosphotungstic acid (PTA) and visualized with a Tecnai G2 Spirit Transmission Electron Microscope operating at 100 kV. Images were collected with a CCD camera Morada 2kx4k.

5.11 Mice immunizations and tumor challenge

Mice immunizations were carried out at 1 or 2 weeks intervals, repeated 3 or 4 times, by i.p. injections with different vaccine's formulations: 1) "Empty" OMVs (derived from BL21(DE3)*ΔompA*(pET21b+) strain*)* 2) Nm-fHbpvIII OMVs (derived from BL21(DE3)*ΔompA*(pET-Nm-fHbp-vIII) strain) 3) Nm-fHbp-M30-vIII OMVs (derived from BL21(DE3)*ΔompA*(pET-Nm-fHbp-M30-vIII) strain) 4) MBP-D8-mFAT1 OMVs (derived from BL21(DE3)*ΔompA*(pET-MBP-D8-mFAT1) strain) 5) TEXs derived from CT26 cell lines. Each sample (20µg) was formulated in a final volume of 200µl of sterile 1X PBS. MBP-D8-mFAT1 OMVs and TEXs were formulated with Alum (Alhydrogel® Adjuvant – Aurogene). Tumor challenge using B16F10EGFRvIII or CT26 cell lines were performed by s.c. injection of 0.5 x 10^5 or 1.5 x 10^5 cells respectively. Tumor growths were then measured during following days using a caliper and mice were sacrificed once tumor volumes exceeded 1500mm^3. Statistical analysis and graphs were processed with Prism 5.0 software (Graphpad). One asterisk: Pval≤0.05; two asterisks: Pval≤0.01; three asterisks: Pval≤0.001.

5.12 ELISA titers

ELISA was performed using Nunc Immobilizer Amino plates (Thermo Fisher Scientific). More specifically, coating was carried out by incubating plates o/n at 4°C with 100 µl of synthetic EGFRvIII or D8-mFAT1 peptides (5 µg/ml), (Genscript). The day after, wells were washed 3 times with TPBS (0.05%

Tween 20 dissolved in PBS, pH 7.4), saturated with 100 µl of 1% BSA dissolved in PBS for 1 hour at 37°C and washed again 3 times with TPBS. Mice sera were threefold serially diluted in TPBS and 0,1% BSA. Serum deriving from mice immunized with “Empty” OMVs was used as negative control. After 3 washes with TPBS, 100 µl of each serum dilution were dispensed in plate wells and incubated 2 hours at 37°C. Wells were subsequently washed 3 times with TPBS and incubated for 1 hour at 37°C with alkaline phosphatase-conjugated goat anti-mouse IgGs at a final dilution of 1:2,000. After triple TPBS wash, 100 µl of Alkaline Phosphatase substrate (Sigma Aldrich) were added to each well and plates were maintained at RT in the dark for 30 minutes. Finally, absorbance was read at 405nm using the M2 Spectramax Reader plate instrument.

Alternatively, TEXs were added to 96-well Maxisorp plates (Thermo Fisher Scientific) in a final concentration of 5 µg/ml in PBS and incubated at RT o/n. The day after, wells were washed 3 times with TPBS, saturated with 100 µl of 1% BSA-PBS for 1 hour at 37°C and washed again 3 times with TPBS. Mice sera were threefold serially diluted in 0,1% BSA-TPBS. After 3 washes with TPBS, 100 µl of each serum dilution were dispensed in plate wells and incubated 2 hours at 37°C. Wells were subsequently washed 3 times with TPBS and incubated for 1 hour at 37°C with alkaline phosphatase-conjugated goat anti-mouse IgGs at a final dilution of 1:2,000. After triple TPBS wash, 100 µl of Alkaline Phosphatase substrate (Sigma Aldrich) were added to each well and plates were maintained at RT in the dark for 30 minutes. Finally absorbance was read at 405nm using the M2 Spectramax Reader plate instrument.

5.13 Flow citometry analysis

B16F10 or CT26 (5×10^4) cells/well were pelletted in 96 U-bottom microplates by centrifugation at 200 x g for 5 minutes at 4°C and incubated for 1 hour at 4°C with pooled serum of mice immunized with MBP-D8-mFAT1 OMVs or with “Empty” OMVs at 1:400 final dilution in PBS. Cells were then washed twice in PBS-5% FBS (FPBS) and incubated 100µl/well with Alexa Fluor 488 (Invitrogen) goat anti mouse at a final dilution 1:200 in PBS for 30 minutes at

4°C in the dark. After 2 washing steps in FPBS, cells were resuspended in 150µl of PBS and were analysed by a FACS-Canto-II flow cytometer (BD Biosciences, San Jose, CA, U.S.) and data were visualized with the FlowJo (Ashland, OR, U.S.) software.

6 REFERENCES

1. WHO. WHO | Cancer. *WHO* (2016). doi:/entity/mediacentre/factsheets/fs297/en/index.html

2. Biemar, F. & Foti, M. Global progress against cancer-challenges and opportunities. *Cancer Biol. Med.* **10,** 183–6 (2013).

3. Society, A. cancer. the Costs of cancer. *ReCALL*

4. Meropol, N. J. & Schulman, K. A. Cost of cancer care: issues and implications. *J. Clin. Oncol.* **25,** 180–6 (2007).

5. Kumar, P. & Moy, B. The cost of cancer care--balancing our duties to patients versus society: are they mutually exclusive? *Oncologist* **18,** 347–9 (2013).

6. Aitken, M. *et al.* Global Oncology Trend Report: A Review of 2015 and Outlook to 2020. *BMC Health Serv. Res.* **10,** 42 (2016).

7. Lizée, G. *et al.* Harnessing the Power of the Immune System to Target Cancer. *Annu. Rev. Med.* **64,** 71–90 (2013).

8. Sell, S. Cancer immunotherapy: Breakthrough or 'deja vu, all over again'? *Tumor Biol.* **39,** 101042831770776 (2017).

9. Atkins, M. B. *et al.* High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: Analysis of 270 patients treated between 1985 and 1993. *J. Clin. Oncol.* **17,** 2105–2116 (1999).

10. Farkona, S., Diamandis, E. P. & Blasutig, I. M. Cancer immunotherapy: the beginning of the end of cancer? *BMC Med.* **14,** 73 (2016).

11. Simpson, A. *et al.* Monoclonal antibodies for the therapy of cancer. *BMC Proc.* **8,** O6 (2014).

12. Wurz, G. T., Kao, C.-J. & DeGregorio, M. W. Novel cancer antigens for personalized immunotherapies: latest evidence and clinical potential. *Ther. Adv. Med. Oncol.* **8,** 4–31 (2015).

13. Parslow, A., Parakh, S., Lee, F.-T., Gan, H. & Scott, A. Antibody–Drug Conjugates for Cancer Therapy. *Biomedicines* **4,** 14 (2016).

14. Sharma, P., Hu-Lieskovan, S., Wargo, J. A. & Ribas, A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. *Cell* **168,** 707–723 (2017).

15. Robert, C. *et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N. Engl. J. Med.* **364,** 2517–2526 (2011).

16. Schadendorf, D. *et al.* Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *J. Clin. Oncol.* **33,** 1889–94 (2015).

17. Zou, W., Wolchok, J. D. & Chen, L. PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: Mechanisms, response biomarkers, and combinations. *Sci. Transl. Med.* **8,** 328rv4 (2016).

18. Sharma, P. & Allison, J. P. Immune checkpoint targeting in cancer therapy: Toward combination strategies with curative potential. *Cell* **161,** 205–214 (2015).

19. Larkin, J. *et al.* Combined Nivolumab and Ipilimumab or Monotherapy in Untreated

Melanoma. *N. Engl. J. Med.* **373,** 23–34 (2015).

20. Sierro, S., Romero, P. & Speiser, D. E. The CD4-like molecule LAG-3, biology and therapeutic applications. *Expert Opin. Ther. Targets* **15,** 91–101 (2011).

21. Tsiatas, M., Mountzios, G. & Curigliano, G. Future perspectives in cancer immunotherapy. *Ann. Transl. Med.* **4,** 273 (2016).

22. Shi, H. *et al.* The status, limitation and improvement of adoptive cellular immunotherapy in advanced urologic malignancies. *Chin. J. Cancer Res.* **27,** 128–137 (2015).

23. Yee, C. Adoptive T-cell therapy for cancer: Boutique therapy or treatment modality? *Clin. Cancer Res.* **19,** 4550–4552 (2013).

24. Rosenberg, S. A. & Restifo, N. P. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science (80-.).* **348,** 62–68 (2015).

25. Kershaw, M. H., Westwood, J. a & Hwu, P. Dual-specific T cells combine proliferation and antitumor activity. *Nat. Biotechnol.* **20,** 1221–7 (2002).

26. Kershaw, M. H. *et al.* A Phase I Study on Adoptive Immunotherapy Using Gene-Modified T Cells for Ovarian Cancer. *Clin. Cancer Res.* **12,** 6106–6115 (2006).

27. Schaller, T. H. & Sampson, J. H. Advances and challenges: dendritic cell vaccination strategies for glioblastoma. *Expert Rev. Vaccines* **584,** 14760584.2016.1218762 (2016).

28. Anguille, S., Smits, E. L., Lion, E., Van Tendeloo, V. F. & Berneman, Z. N. Clinical use of dendritic cells for cancer therapy. *The Lancet Oncology* **15,** (2014).

29. Lee, S. & Margolin, K. Cytokines in cancer immunotherapy. *Cancers* **3,** 3856–3893 (2011).

30. Sim, G. C. & Radvanyi, L. The IL-2 cytokine family in cancer immunotherapy. *Cytokine and Growth Factor Reviews* **25,** 377–390 (2014).

31. 1. Aas, D. E. L. H. et al. Stable transduction of quiescent CD34+ CD38- human hematopoietic cells by HIV-1-based lentiviral vectors. PNAS 96, 2988–2993 (1999). *et al.* Brief report Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood* **116,** 4099–4103 (2010).

32. Cappuzzello, E., Sommaggio, R., Zanovello, P. & Rosato, A. Cytokine & Growth Factor Reviews Cytokines for the induction of antitumor effectors : The paradigm of Cytokine-Induced Killer (CIK) cells. *Cytokine Growth Factor Rev.* 1–7 (2017). doi:10.1016/j.cytogfr.2017.06.003

33. Yu, P., Steel, J. C., Zhang, M., Morris, J. C. & Waldmann, T. A. Simultaneous blockade of multiple immune system inhibitory checkpoints enhances antitumor activity mediated by interleukin-15 in a murine metastatic colon carcinoma model. *Clin. Cancer Res.* **16,** 6019–6028 (2010).

34. Brandt, K., Bulfone-Paus, S., Foster, D. C. & Rückert, R. Interleukin-21 inhibits dendritic cell activation and maturation. *Blood* **102,** 4090–4098 (2003).

35. Valletta, D. *et al.* Regulation and function of the atypical cadherin FAT1 in hepatocellular carcinoma. *Carcinogenesis* **35,** 1407–1415 (2014).

36. Schreiber, R. D., Old, L. J. & Smyth, M. J. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. *Science (80-.).* **331,** 1565–1570 (2011).

37. Buonaguro, L., Petrizzo, A., Tornesello, M. L. & Buonaguro, F. M. Translating tumor antigens into cancer vaccines. *Clinical and Vaccine Immunology* **18,** 23–34 (2011).

38. Klebanoff, C. A., Acquavella, N., Yu, Z. & Restifo, N. P. Therapeutic cancer vaccines: Are we there yet? *Immunol. Rev.* **239,** 27–44 (2011).

39. Pileri, P. *et al.* FAT1: a potential target for monoclonal antibody therapy in colon cancer. *Br. J. Cancer* **115,** 40–51 (2016).

40. Rizvi, N. A. *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science (80-.).* **348,** 124–128 (2015).

41. Van Allen, E. M. *et al.* Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science (80-.).* **350,** 207–211 (2015).

42. Snyder, A. *et al.* Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N. Engl. J. Med.* **371,** 2189–2199 (2014).

43. Théry, C. Exosomes: secreted vesicles and intercellular communications. *Biol. Reports* **33410,** 15–3 (2011).

44. Théry, C., Zitvogel, L. & Amigorena, S. Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* **2,** 569–579 (2002).

45. Yu, S., Cao, H., Shen, B. & Feng, J. Tumor-derived exosomes in cancer progression and treatment failure. *Oncotarget* **6,** 37151–37168 (2015).

46. Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J. & Raposo, G. The biogenesis and functions of exosomes. *Traffic* **3,** 321–330 (2002).

47. Abels, E. R. & Breakefield, X. O. Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake. *Cell. Mol. Neurobiol.* **36,** 301–312 (2016).

48. Pan, B. T., Teng, K., Wu, C., Adam, M. & Johnstone, R. M. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J. Cell Biol.* **101,** 942–948 (1985).

49. Prendergast, G. C. & Jaffee, E. M. *Introduction*. (2013).

50. Kalra, H. *et al.* Vesiclepedia: A Compendium for Extracellular Vesicles with Continuous Community Annotation. *PLoS Biol.* **10,** (2012).

51. Kim, D.-K. *et al.* EVpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles. *J. Extracell. vesicles* **2,** 1–7 (2013).

52. Simpson, R. J., Kalra, H. & Mathivanan, S. ExoCarta as a resource for exosomal research. *J. Extracell. Vesicles* **1,** 1–6 (2012).

53. Yang, C. & Robbins, P. D. The roles of tumor-derived exosomes in cancer pathogenesis. *Clin. Dev. Immunol.* **2011,** (2011).

54. Kunigelis, K. E. & Graner, M. W. *The Dichotomy of Tumor Exosomes (TEX) in Cancer Immunity: Is It All in the ConTEXt?* (2015). doi:10.3390/vaccines3041019

55. Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L. & Turbide, C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* **262,** 9412–9420 (1987).

56. Beach, A., Zhang, H., Ratajczak, M. Z. & Kakar, S. S. Exosomes : an overview of biogenesis , composition and role in ovarian cancer Exosomes : an overview of biogenesis , composition and role in ovarian cancer. *J. Ovarian Res.* **7,** 1–10 (2014).

57. Raposo, G. & Stoorvogel, W. Extracellular vesicles: Exosomes, microvesicles, and friends. *Journal of Cell Biology* **200,** 373–383 (2013).

58. Silva, J. *et al.* Analysis of exosome release and its prognostic value in human colorectal cancer. *Genes. Chromosomes Cancer* **51,** 409–18 (2012).

59. Rabinowits, G., Gerçel-Taylor, C., Day, J. M., Taylor, D. D. & Kloecker, G. H. Exosomal microRNA: a diagnostic marker for lung cancer. *Clin. Lung Cancer* **10,** 42–6 (2009).

60. Mahmoodzadeh Hosseini, H., Halabian, R., Amin, M. & Imani Fooladi, A. A. Texosome-based drug delivery system for cancer therapy: from past to present. *Cancer Biol. Med.* **12,** 150–62 (2015).

61. Spier, R. E. Multivalent vaccines: Prospects and challenges. *Folia Microbiol. (Praha).* **42,** 105–112 (1997).

62. Rao, Q. *et al.* Tumor-derived exosomes elicit tumor suppression in murine hepatocellular carcinoma models and humans in vitro. *Hepatology* **64,** 456–472 (2016).

63. Zech, D., Rana, S., Büchler, M. W. & Zöller, M. Tumor-exosomes and leukocyte activation: an ambivalent crosstalk. *Cell Commun. Signal.* **10,** 37 (2012).

64. Zeelenberg, I. S. *et al.* Antigen Localization Controls T Cell-Mediated Tumor Immunity. *J. Immunol.* **187,** 1281–1288 (2011).

65. Khalil, A. A., Kabapy, N. F., Deraz, S. F. & Smith, C. Heat shock proteins in oncology: Diagnostic biomarkers or therapeutic targets? *Biochimica et Biophysica Acta - Reviews on Cancer* **1816,** 89–104 (2011).

66. Chen, T., Guo, J., Yang, M., Zhu, X. & Cao, X. Chemokine-Containing Exosomes Are Released from Heat-Stressed Tumor Cells via Lipid Raft-Dependent Pathway and Act as Efficient Tumor Vaccine. *J. Immunol.* **186,** 2219–2228 (2011).

67. Hurwitz, M. D. *et al.* Radiation therapy induces circulating serum Hsp72 in patients with prostate cancer. *Radiother. Oncol.* **95,** 350–358 (2010).

68. Gu, X., Erb, U., Büchler, M. W. & Zöller, M. Improved vaccine efficacy of tumor exosome compared to tumor lysate loaded dendritic cells in mice. *Int. J. Cancer* **136,** E74–E84 (2015).

69. Morishita, M., Takahashi, Y., Matsumoto, A., Nishikawa, M. & Takakura, Y. Exosome-based tumor antigens adjuvant co-delivery utilizing genetically engineered tumor cell-derived exosomes with immunostimulatory CpG DNA. *Biomaterials* **111,** 55–65 (2016).

70. Lener, T. *et al.* Applying extracellular vesicles based therapeutics in clinical trials - an ISEV position paper. *J. Extracell. Vesicles* **4,** 1–31 (2015).

71. Dai, S. *et al.* Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. *Mol. Ther.* **16,** 782–790 (2008).

72. Tan, A., Rajadas, J. & Seifalian, A. M. Exosomes as nano-theranostic delivery platforms for gene therapy. *Advanced Drug Delivery Reviews* **65,** 357–367 (2013).

73. Temizoz, B., Kuroda, E. & Ishii, K. J. Vaccine adjuvants as potential cancer immunotherapeutics. *Int. Immunol.* **28,** 329–338 (2016).

74. Hartman, L. L. *et al.* Pediatric phase II trials of poly-ICLC in the management of newly diagnosed and recurrent brain tumors. *J Pediatr Hematol Oncol* **36,** 451–457 (2014).

75. Cluff, C. W. Monophosphoryl lipid a (MPL) as an adjuvant for anti-cancer vaccines: Clinical results. *Adv. Exp. Med. Biol.* **667,** 111–123 (2009).

76. Gillessen, S. *et al.* CD1d-restricted T cells regulate dendritic cell function and antitumor immunity in a granulocyte-macrophage colony-stimulating factor-dependent fashion. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 8874–9 (2003).

77. Cheng, Y. C. *et al.* Addition of GM-CSF to trastuzumab stabilises disease in trastuzumab-resistant HER2+ metastatic breast cancer patients. *Br. J. Cancer* **103,** 1331–1334 (2010).

78. Wiedermann, U. *et al.* A virosomal formulated Her-2/neu multi-peptide vaccine induces Her-2/neu-specific immune responses in patients with metastatic breast cancer: A phase i study. *Breast Cancer Res. Treat.* **119,** 673–683 (2010).

79. Neelapu, S. S. *et al.* Human autologous tumor-specific T-cell responses induced by liposomal delivery of a lymphoma antigen. *Clin. Cancer Res.* **10,** 8309–8317 (2004).

80. Chen, Q. *et al.* Immunodominant CD4+ responses identified in a patient vaccinated with full-length NY-ESO-1 formulated with ISCOMATRIX adjuvant. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 9363–8 (2004).

81. HogenEsch, H. Mechanism of immunopotentiation and safety of aluminum adjuvants. *Front. Immunol.* **3,** (2012).

82. Chiang, C. L.-L., Kandalaft, L. E. & Coukos, G. Adjuvants for enhancing the immunogenicity of whole tumor cell vaccines. *Int. Rev. Immunol.* **30,** 150–82 (2011).

83. Aucouturier, J., Dupuis, L., Deville, S., Ascarateil, S. & Ganne, V. Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. *Expert Rev. Vaccines* **1,** 111–118 (2002).

84. Vinageras, E. N. *et al.* Phase II randomized controlled trial of an epidermal growth factor vaccine in advanced non-small-cell lung cancer. *J. Clin. Oncol.* **26,** 1452–1458 (2008).

85. Yang, M. *et al.* MF59 formulated with CpG ODN as a potent adjuvant of recombinant HSP65-MUC1 for inducing anti-MUC1 + tumor immunity in mice. *Int. Immunopharmacol.* **13,** 408–416 (2012).

86. Kulp, A. & Kuehn, M. J. Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles. *Annu Rev Microbiol* **64,** 163–184 (2010).

87. O'Donoghue, E. J. & Krachler, A. M. Mechanisms of outer membrane vesicle entry into host cells. *Cell. Microbiol.* **18,** 1508–1517 (2016).

88. Giuliani, M. M. *et al.* A universal vaccine for serogroup B meningococcus. *Proc. Natl. Acad. Sci.* **103,** 10834–10839 (2006).

89. Fantappiè, L. *et al.* Antibody-mediated immunity induced by engineered Escherichia coli OMVs carrying heterologous antigens in their lumen. **1,** 1–14 (2014).

90. Gerritzen, M. J., Martens, D. E., Wijffels, R. H., van der Pol, L. & Stork, M. Bioengineering bacterial outer membrane vesicles as vaccine platform. *Biotechnol. Adv.* (2017). doi:10.1016/j.biotechadv.2017.05.003

91. Kesty, N. C. & Kuehn, M. J. Incorporation of heterologous outer membrane and periplasmic proteins into Escherichia coli outer membrane vesicles. *J. Biol. Chem.* **279,** 2069–76 (2004).

92. Fantappiè, L. *et al.* Some Gram-negative Lipoproteins Keep Their Surface Topology When Transplanted from One Species to Another and Deliver Foreign Polypeptides to the Bacterial Surface. *Mol. Cell. Proteomics* **16,** 1348–1364 (2017).

93. Berlanda Scorza, F. *et al.* High yield production process for Shigella outer membrane particles. *PLoS One* **7,** (2012).

94. Butterfield, L. H. Cancer vaccines. *BMJ* **350,** h988 (2015).

95. Sayour, E. J. & Mitchell, D. A. Manipulation of Innate and Adaptive Immunity through Cancer Vaccines. *J. Immunol. Res.* **2017,** (2017).

96. Guo, C. *et al.* Therapeutic cancer vaccines. Past, present, and future. *Adv. Cancer Res.* **119,** 421–475 (2013).

97. Ross, M. I. *et al.* Patterns of durable response with intralesional talimogene laherparepvec (T-VEC): Results from a phase III trial in patients with stage IIIb-IV melanoma. *J. Clin. Oncol.* **32,** 9026 (2014).

98. Keller, B. A. & Bell, J. C. Oncolytic viruses immunotherapeutics on the rise. *J. Mol. Med.* **94,** 979–991 (2016).

99. Fukuhara, H., Ino, Y. & Todo, T. Oncolytic virus therapy: A new era of cancer treatment at dawn. *Cancer Sci.* **107,** 1373–1379 (2016).

100. Flaherty, K. T. *et al.* Inhibition of Mutated, Activated BRAF in Metastatic Melanoma. *N. Engl. J. Med.* **363,** 809–819 (2010).

101. Kreiter, S., Castle, J. C., Türeci, O. & Sahin, U. Targeting the tumor mutanome for personalized vaccination therapy. *Oncoimmunology* **1,** 768–769 (2012).

102. Stratton MR. Exploring the Genomes of Cancer Cells: Progress and Promise. *Science (80-.).* **331,** 1553–8 (2011).

103. Sahin, U. *et al.* Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nat. Publ. Gr.* (2017). doi:10.1038/nature23003

104. Ott, P. A. *et al.* An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* **547,** 217–221 (2017).

105. Del Vecchio, C. a, Li, G. & Wong, A. J. Targeting EGF receptor variant III: tumor-specific peptide vaccination for malignant gliomas. *Expert Rev. Vaccines* **11,** 133–44 (2012).

106. Heimberger, A. B. *et al.* Epidermal growth factor receptor VIII peptide vaccination is efficacious against established intracerebral tumors. *Clin. Cancer Res.* **9,** 4247–4254 (2003).

107. Kreiter, S. *et al.* Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* **520,** 692–696 (2015).

108. Dunne, J. *et al.* Molecular cloning and tissue expression of FAT, the human homologue of the Drosophila fat gene that is located on chromosome 4q34-q35 and encodes a putative adhesion molecule. *Genomics* **30,** 207–223 (1995).

109. Mitchell, J. P., Court, J., Mason, M. D., Tabi, Z. & Clayton, A. Increased exosome production from tumour cell cultures using the Integra CELLine Culture System. *J. Immunol. Methods* **335,** 98–105 (2008).

110. Kranz, L. M. *et al.* Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. *Nature* **534,** 396–401 (2016).

111. Sockolosky, J. T. *et al.* Durable antitumor responses to CD47 blockade require adaptive immune stimulation. *Proc. Natl. Acad. Sci.* **113,** 201604268 (2016).

112. Klock, H. E. & Lesley, S. A. The polymerase incomplete primer extension (PIPE) method applied to high-throughput cloning and site-directed mutagenesis. *Methods Mol. Biol.* **498,** 91–103 (2009).

113. Piatkevich, K. D. & Verkhusha, V. V. *Guide to red fluorescent proteins and biosensors for flow cytometry*. *Methods in Cell Biology* **102,** (2011).

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