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RESEARCH ARTICLE

Engineering New Immunotherapies against Cancer using Bacterial Outer Membrane Vesicles and Supported by Preclinical Data

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ABSTRACT

Cancer remains a serious challenge to public health, with breast cancer, lung, and colorectal cancers predominating in both incidence and deaths worldwide. Significant advances have been made in the therapeutic treatment and resolution of non-solid tumors using immune checkpoint inhibitors (ICI) and chimeric antigen receptor (CAR) T cell therapy to break immune tolerance and initiate tumor clearance. However, these innovative strategies have enjoyed only limited success with solid tumors, especially in late-stage cancers in which tumor size is large. An immunosuppressive tumor microenvironment (TME) that surrounds and protects solid tumors significantly confounds the ability of the host immune system to target and eliminate tumor tissue. Novel technologies using nanomedicines have begun to yield promising results by penetrating into the microenvironment to stimulate innate immunity and induce trafficking of activated antigen presenting cells to regional lymph nodes, ultimately leading to tumor-specific adaptive immune responses. One type of nanomedicine that is generating increasing enthusiasm in the field of immunotherapy are bacterial outer membrane vesicles (OMVs) that can be genetically engineered to surface-express tumor-associated antigens; the resulting recombinant OMVs (rOMVs) can then be purified as immunotherapeutic vaccines. Recent data from experimental animal models have demonstrated remarkable efficacy in tumor challenge models. Such promising experiments suggest the possibility of translating these novel strategies into success with solid tumors in clinical trials. In this review, we will summarize current research using purified rOMVs as immunotherapeutic vaccines and further discuss potential obstacles that still need to be adequately addressed to ensure success in human trials.

INTRODUCTION

Cancer continues to pose tremendous challenges to public health throughout the world. Global estimates of cancer incidence from 185 countries included in the GLOBOCAN 2020 database list female breast cancer, lung, colorectal, and prostate cancer as the most commonly diagnosed cancers; lung cancer was the leading cause of death, followed by colorectal, liver, stomach, and female breast cancers.[1] Within the United States, estimates reported by the American Cancer Institute for cancer incidence and mortality reflect the trends seen worldwide, with breast, prostate, lung, and colorectal cancer predominating in both incidence and deaths.[2] Although significant advances have been made in the therapeutic treatment and resolution of non-solid tumors, success in the treatment of solid tumors still has not been able to move beyond the standard of care that currently includes surgery, chemotherapy, radiation, and/or combinations of these modalities. Until recently, less aggressive treatment strategies attempting to exploit the host immune system to clear tumor tissues seemed out of reach, but the recent success of immune checkpoint inhibitors (ICI) that activate tumor-specific T cells has rekindled significant interest in immunotherapeutic approaches for the treatment of cancer.[3] However, ICI strategies and other recent T cell-targeting immune strategies (such as CAR-T cell therapy), while yielding success with some tumors, have not translated into significant improvements in their application to solid tumors, especially in late-stage cancers in which tumor size is large.[4-6] An immunosuppressive tumor microenvironment (TME) surrounds solid tumors and significantly confounds the ability of the host adaptive immune system to target and eliminate tumor tissue. Clinical studies have thus demonstrated only incremental steps in the development of immunotherapeutic interventions to treat either primary solid tumors or to prevent the metastasis of remnant tumor cells persisting post primary treatment.

In this review, we will briefly summarize some of the key aspects of tumor biology and selected mechanisms by which actively growing tumors subvert the normal immunosurveillance activities of an otherwise fully functional immune system. In view of the diversity of cancers and the heterogeneity of cell types involved, we have tried to focus on mechanisms common to most types of cancers. We then describe various therapeutic approaches to combatting tumor progression and metastasis that have been examined for efficacy in experimental animal models, and then explore an emerging new approach to therapeutic immunization against tumors involving outer membrane vesicles isolated from bacteria and engineered as tumor-specific vaccines.

1. Tumor biology, the microenvironment, and immunosuppression

The gradual transformation of normal cells into tumor cells involves the accumulation of various mutations in metabolic and regulatory genes that enable unrestricted growth of the resulting cells. The survival of a nascent tumor depends on this rapidly proliferating aggregation of cells acquiring multiple critical capabilities of growth and metabolism including: 1] the capacity to elicit both proliferative signaling and replicative immortality; 2] evasion of growth suppressers while sustaining replicative immortality; 3] secretion of factors inducing the formation of new blood vessels to supply oxygen and nutrients to a rapidly growing cell mass; 4] rapid adjustment of cell physiology and metabolism to a rapidly changing microenvironment; 5] avoiding immunosurveillance and tumor-targeted eradication; and 6] upregulation of local tissue invasion and metastasis of tumor cells to distant anatomical sites. Many of the these metabolic adjustments and new-found functionalities are enabled by intracellular genomic instability as well as infiltration of innate and adaptive cells that are recruited and reprogrammed for the secretion of cytokines that promote tumor growth.[7]

During the early stages of tumor formation, occurring in the presence of a normally and fully functioning host immune system, tumors also progress through a series of cellular remodeling steps (collectively referred to as “immunoediting”) that eventually lead to escape from immunosurveillance.[7, 8] Initially, tumor cells are subject to elimination by both innate and adaptive immune effector cells. However, the genetic instability of tumor cells allows them to shed many of the surface signaling proteins, including major histocompatibility I (MHC I) and over-expression of protein antigens typically either not expressed in normally differentiated tissues, or arising again from mutation of appropriately expressed proteins leading to “neoantigens”.[8, 9] Such a shift in antigen expression profiles allows tumors to escape immunosurveillance. However, the steadily growing tumor is still unable to sustain itself without creating an environment, referred to as a tumor microenvironment (TME), that not only provides the structural support and energy required for growth, but also provides additional shielding of the growing tumor from immune surveillance through direct interference of immune cell infiltration (immune exclusion) and interruption of anti-tumor effector functions of resident immune cells (immune failure).[7, 10-

[12] This has led to tumors being referred to as "cold" or "hot" tumors. Cold tumors are refractory to immunotherapy characterized by immune evasion and exclusion (**Figure 1A**); hot tumors are characterized by pre-existing tumor infiltration with activated tumor-specific immune cells that can respond to appropriate immunotherapeutic vaccination (**Figure 1B**).[13]

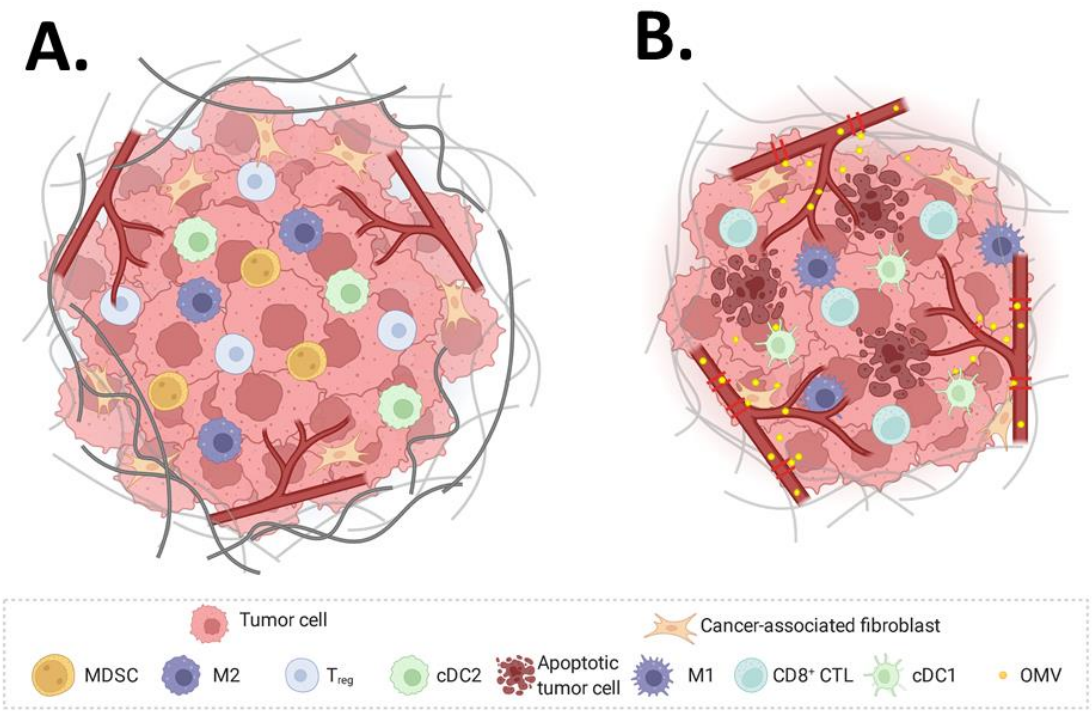

Figure 1. Schematic representation of the tumorigenic environment of an established tumor (panel A) versus a tumoricidal environment for a tumor in remission (panel B). Selected cells populating the tumor microenvironment are represented here and described further in the text. Small parallel bars in blood vessels represent the fenestrations of small rapidly growing tumors. Small yellow circles represent OMVs. Gray fibrils represent the extracellular matrix.

The microenvironment can be infiltrated with both innate and adaptive immune cells. Innate immune cells include macrophages, natural killer (NK) cells, natural killer T cells (NKT), and dendritic cells (DCs).[7, 8, 12-14] Many innate immune cells can be functionally categorized into two broadly defined types depending on whether they display anti-tumor activities or tumorigenic activities. Tumor cells can elicit cytokines that reprogram innate immune cells from type 1 to type 2 phenotypes to foster tumor growth and survival.[14, 15] For example, tumor-associated macrophages (TAMs) can be either tumor-suppressing M1 cells or tumor-supportive M2 cells. M1 cells can secrete interleukin 12 (IL-12) which promotes the link between innate and adaptive tumor-specific immunity; IL-12 also induces the production of interferon γ (IFN-γ), which in turn, plays a role in the significant proliferation of T cell helper 1 (Th1) and in modulating cytotoxic T cells (CTLs) and NK cytotoxic activity, including the expansion and survival of activated T-cells and NK cells[7]. IFN-γ acting on bystander M1 polarized macrophages dramatically increases their sensitivity to lipopolysaccharides and other toll-like receptor (TLR) ligands and decreases sensitivity to anti-inflammatory interleukins including IL-10.[16] M2 macrophages secrete IL-10 which suppresses local tumoricidal activity in the TME, while also promoting tissue remodeling functions that support tumor growth.[7, 8, 14]

Similarly, NK cells can be either NK1 or NK2 cells. NK cells do not utilize T cell receptors or major histocompatibility complex I (MHC-I) molecules to identify and kill tumor cells. Similar to NK cells, NKT1 and NKT2 cells have been described but unlike NK

cells these cells have distinct T cell repertoires and recognize antigen in an MHC-I like cluster of differentiation (CD)1d-restricted context; NKT1s are typically tumor-suppressing, while NKT2 cells predominantly display protumor immunoregulatory activity.[12, 14] Finally, DCs can be grouped into cDC1s and cDC2s. cDC1s are the only population that mediate the transport of solid tumor antigens from TME to tumor draining lymph nodes for cross-priming of CD8 T cells.[10] cDC2s are not as efficient at cross-presentation[7] and are implicated in suppression of anti-tumor responses.

Adaptive cells in the TME include CD4+ and CD8+ T cells as well as antibody-producing B cells. As with innate immunity, adaptive immune cells can also be categorized into tumor active and suppressive subsets, and transitions between the two phenotypes can again be controlled by tumor cells and the TME. CD4+ cells can be Th1 or Th2 CD4+ helper cells; it is currently believed that Th1 cells are the principal subset along with NK cells and cytotoxic CD8+ T cells involved in eradicating tumors[7, 13]; CD4+ Th2 cells along with CD4+ T_{reg} cells have anti-tumor suppression functions.[17] CD4+ Th2 cells can release IL-4 and IL-13 which promote the polarization of tumor-associated macrophages towards the M2 phenotype.[12] Multiple subsets of CD8+ T cells have also been identified including Tc1s and Tc2s; a low Tc1/Tc2 ratio has been correlated with a poor prognosis in cancer patients.[7] B cells promote anti-tumor activity by producing tumor-specific antibodies that facilitate opsonization of tumor cells by DCs, activation of the complement cascade, and induction of antibody-dependent cellular cytotoxicity by NK cells.[7]

In addition to the 1 / 2-type polarization of immune cells, tumors can escape immunosurveillance by producing cytokines that suppress resident macrophages and recruit still other immune cells including myeloid-derived suppressor cells (MDSC), and regulatory T cells (T_{reg}s) that become increasingly prevalent in the tumor microenvironment and draining lymph nodes of the tumor, while levels of antitumoral CTLs and NK cells are reduced.[8, 10, 15] MDSCs are immature myeloid cells that interfere with tumor-specific immunity and are recruited by tumors that produce granulocyte-macrophage colony-stimulating factor (GM-CSF).[8] MDSCs can also promote T_{reg}s which in turn display an immunosuppressive phenotype that can work together with MDSCs to suppress the expansion of activated CD4+ T helper and CD8+ cytotoxic T cells and enable tumor progression.[8, 9] In addition, MDSCs can interrupt DC maturation through secretion of IL-10 and over-expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) which binds to the co-stimulatory cluster of differentiation 80 (CD80) of DCs[9].

Downregulation of DC maturation, antigen presentation, and costimulatory molecule expression is a key mechanism in achieving tumor immune evasion. Since activated DCs are key to the activation of CTLs through cross-priming of intracellularly processed tumor antigens, inhibition of DC activation therefore reduces direct targeting of tumors. M2 cells, MDSCs, and T_{reg}s have been shown to inhibit DC maturation through secretion of the cytokines IL-6 and/or IL-10 which interferes with DC maturation and blocks the stimulation of tumor-reactive M1 macrophages[7, 10, 13]; presentation of antigens to T cells in the absence of DC maturation after uptake of tumor antigens leads to T cell tolerance.[8, 18] In addition, activated T cells can be blocked in migrating from regional lymph nodes to the tumor through downregulation of chemokine and cytokine gradients by cancer-associated fibroblasts (CAFs). CAFs may also impair DC trafficking from lymph nodes.[13]

2. Conventional approaches to tumor immunotherapy.

Taken together, the discussion above describes numerous challenging obstacles that a therapeutic vaccine must overcome in order to activate the cytotoxic immune cells needed for solid tumor eradication. It therefore becomes evident that for an optimum immune response against a tumor to occur, the immune system must provide mature tumor-specific cDC1s, and activated M1 macrophages, NK1 cells, Th1 cells, and CD8+ T cells[8, 17]; in addition, immune suppression in the TME by M2 macrophages, MDSCs, and T_{reg}s must be reduced to homeostasis levels.[17] Recent efforts in tumor immunotherapy have therefore focused on immunomodulation within the TME using various therapeutic vaccine strategies.[18] However, results from numerous clinical trials illustrate the complexity of tumor biology, the diversity of patient-specific immunity, and the challenge of exclusively targeting tumor cells while avoiding off-target reactogenicity. These limitations have collectively contributed to only a modest efficacy in clinical trials through failure to elicit sustained tumor-specific cytotoxic responses and durable T cell memory against relapse and metastasis.[13] Given the inaccessibility of tumor tissue and the inherent genetic instability of tumor cells that can rapidly alter surface antigens to evade active immunosurveillance by immune effector cells, disruption of the immunosuppressive environment of the TME becomes a highly promising strategy to activate anergic innate

immune cells and trigger robust adaptive responses through tumor-specific cytotoxicity.

Surgical intervention to remove advanced-stage localized solid tumors immediately decreases immunosuppression, opening the way to immunotherapeutic vaccination against the risk of tumor relapse or metastasis of any remaining tumor cells to distant locations.[8, 19] Surgical interventions are typically followed up with chemotherapy and/or radiation treatments to eradicate any remaining tumor cells. Chemotherapy and radiation treatments can both be potent inducers of anticancer immunity against dead or dying tumoral cells through release of damage-associated molecular patterns (DAMPs) that activate surrounding antigen presenting cells in the absence of any additional immune adjuvants.[12, 13, 16] This pathway leading to the stimulation of innate immunity against tumor cells is referred to as "immunogenic cell death" (ICD). During ICD, dying cancer cells release three types of intracellular DAMPs including the surface exposed chaperone calreticulin (CRT), adenosine triphosphate (ATP), and endogenous toll-like receptor agonists that are potent activators of DCs.[9, 12, 20]

Surface presentation of CRT is a primary determinant for attracting DCs to dying cancer cells through binding of CRT to the cell surface receptor CD91 and subsequent phagocytosis of tumor cells.[12, 16] Activation of DCs by CRT-CD91 binding induces the production of IL-6 and tumor necrosis factors (TNF) by DCs which, in turn facilitates the repolarization of protumorigenic Th2 cells to tumor-suppressive Th1 T cells.[20, 21] Release of intracellular adenosine triphosphate (ATP) from dying or dead tumor cells functions as a short-range "find me" alarmone that attracts antigen presenting DC cells to the necrotic tumor environment and enhances activation.[20] And finally, release the intracellular TLR4 agonist high-mobility group box 1 (HMGB1) protein is also a powerful inducer of DC-mediated anti-tumor immunity; activation of DCs through extracellular HMGB1 in surrounding tumor tissue binding to the TLR4 receptor of DCs significantly enhances efficient antigen presentation of tumor antigens while suppressing immunosuppressive T_{reg} cells.[12, 20] Once activated through ICD, DCs quickly differentiate into mature antigen presenting cells expressing additional co-stimulatory signals required for the efficient activation of tumor-specific T cells during antigen presentation, accompanied by increased secretion of IL-6 and reduction in immunosuppressive IL-10 from surrounding immune cells.[16]

Activated cDC1 cells responding to chemotherapy or radiation-induced ICD can drain through the lymphatic system into regional lymph nodes to elicit T cell-mediated antitumor responses through the cross-presentation of tumor antigens in the context of MHC I to CD8+ T cells to elicit tumoricidal CTLs.[16] Regional lymph nodes present the perfect mechanism for bringing rare precursor T cells, with T cell receptors capable of recognizing tumor antigens, into contact with tumor-specific matured cDC1s presenting antigen along with the necessary co-stimulatory surface receptors to stimulate tumor-specific cytotoxic effector functions and avoid the generation of tolerance.[22, 23] Naïve systemic T cells and B cells recirculate through lymph nodes, increasing the probability that antigen-specific immune cells will be stimulated by cognate dendritic cells[24]; activation of these immune cells is limited by the availability of activated DCs presenting tumor antigens. Therefore, lymph nodes are a critical staging area orchestrating the efficient interaction between innate and adaptive immunity, resulting not only in appropriate effector responses but also importantly the generation of tumor-specific memory cells.[23, 25]

3. Use of nanomedicines in tumor treatment.

Another potentially effective way to enhance tumor eradication following chemotherapeutic and/or radiation adjuvant therapies is by taking advantage of the potential priming of the immune system through ICD by administration of a tumor-specific therapeutic vaccine as a booster to raise tumor-specific adaptive immunity and expand memory. Numerous conventional therapeutic vaccines targeting a variety of solid and non-solid tumors have achieved at least some level of promise in clinical trials over the past decade.[8, 13, 18] However, a promising new approach involving nanomedicine platforms has generated significant enthusiasm in recent years. Nanomedicine can be defined as any therapeutic modality (nanostructure) employed for the treatment of disease that has a physical dimension less than 1000 nm (i.e.1 mm). In this regard, nanoparticles are typically smaller than ~100nm [ref 6]. Virus-like particles (VLPs) and small liposomes are typically smaller than ~200 nm [refs26, 27]; in comparison, viruses are typically smaller than 350 nm, bacteria range in size from 1 – 5 μm, and T cells range from 7 – 20 μm. Nanomedicines can be used to avoid systemic side effects and specifically target cancer cells with cytotoxic chemotherapeutic drugs to induce immunogenic cell death (ICD), triggering the release of tumor antigens and DAMPs which in turn can prime the activation of antigen presenting cells (APCs) and consequently, promote T cell antitumoral activities.[19] Nanomedicines in the form of engineered

nanoparticles, VLPs, or small liposomes can also be administered parenterally and then traffick through the lymphatic system into regional lymph nodes, encountering and activating antigen presenting cells including dendritic cells[22, 28]. Interestingly, cross-presentation to T cells of peptides derived from particulate antigens occurs much more readily than cross-presentation of peptides derived from soluble antigens.[27]

Trafficking of parenterally administered therapeutic nanomedicines into lymph nodes can occur by passive diffusion through lymphatic endothelial cell junctions or directly by intravasation into the lumen of lymph vessels by phagocytic immune cells such as Langerhans cells (for intradermal and subcutaneous immunization) or dendritic cells (for intramuscular administration).[29] The efficiency of passive trafficking of nanomedicines from the injection site into regional lymph nodes is critically dependent on the size of the particles constituting the vaccine. 200 nm pores in the walls of the lymphatic vessels allow the direct diffusion of smaller 10-80 nm nanoparticles; nanoparticles < 50 nm in diameter are reported to be phagocytosed by draining lymph node dendritic cells within 24-48 hours of administration.[25] Particles larger than ~100 nm are often trapped in the extracellular matrix at the injection site and require phagocytosis and trafficking by APCs to reach regional lymph nodes[22, 25, 27, 30]; the transport of particles that are larger than 200−500nm requires phagocytosis and trafficking by DCs and takes approximately 24 hours for them to arrive in lymph nodes.[27] Vaccine material that reaches lymph nodes without being phagocytosed flushes through the lymph node and into the blood stream through the thoracic duct, allowing for systemic distribution of remaining vaccine to potentially elicit a secondary immune response.[24]

4. Outer membrane vesicles and tumor immunotherapy.

One type of nanomedicine that is gaining increased interest in the field of tumor interventions, due to robust immunogenicity and relative ease of manufacture, is the bacterial outer membrane vesicle (OMV). Bacterial OMVs are small relatively spherical particles originating from the outer membrane of Gram-negative bacteria that typically range from 30-250 nm in size[31, 32]. OMVs are spontaneously exported off the surface of the bacteria by evagination of the outer membrane, and consequently contain most of the components normally associated with the bacterial outer membrane, including lipopolysaccharide (also called endotoxin and a potent TLR4 agonist), various porins, lipoproteins, and other surface expressed transmembrane proteins. It is precisely because surface membrane proteins are exported on the surface of emerging vesicles that OMVs have become attractive vaccine platforms for use as therapeutic vaccines against tumors.

Perhaps not surprisingly, numerous technologies and strategies are now available that permit the efficient genetic engineering of bacterial OMVs such that engineered cytoplasmic expression of tumor-specific antigens can be efficiently displayed on the outer surface of recombinant vesicles (rOMVs) in the context of the powerful TLR4 agonist lipopolysaccharide (LPS). Transport of cytoplasmically expressed tumor antigens to the surface of the host bacterium can be accomplished by engineering protein fusions of the tumor antigen to transporter proteins that are naturally transported to the outer membrane of the host bacterium. An efficient and versatile transport protein that has been extensively exploited for this purpose is the **c**yto**ly**sin **A** (ClyA) protein from *Salmonella enterica* serovar Typhi. ClyA was first described by Wallace et al.[33], who also reported the crystal structure for the homologous hemolysin E (HlyE) from *E. coli*. HlyE is a kinked rod-shaped 35 kDa molecule with a hydrophobic 27 residue transmembrane region comprising one terminus of the folded molecule. HlyE is exported into the surrounding medium, but is not exported by any of the known export pathways. Surprisingly, Wai et al.[34] showed that ClyA is instead exported via outer membrane vesicles. The over-expression of ClyA has been exploited to efficiently produce outer membrane vesicles. In addition, it has been reported that foreign protein domains can be genetically fused to either the amino- or carboxyl-terminus of ClyA, leading to the production of vesicles in which the foreign fusion partner is displayed on the outside of the exported vesicles.[35-37] This technology has now been used by several groups to export rOMVs which not only display foreign tumor proteins on their surface but also retain immunogenicity and elicit tumor-specific immunity in experimental tumor challenge models (as discussed in more detail below).

Another novel approach that is finding increasing popularity in the engineering of rOMVs is referred to as the Plug-and-Display antigen expression platform. This remarkably versatile platform allows for the export of a single species of rOMV to which any partner antigen can be covalently attached to the rOMV surface without any further modification of the basic rOMV. The system is based on an elegant Spy-Tag bipartite coupling chemistry, derived from the immunoglobulin-like collagen adhesion domain of the *Streptococcus pyogenes* protein CnaB2, in which half of

this domain is engineered onto an acceptor protein anchored to rOMVs and the other reactive half is engineered as a fusion to the targeted foreign antigen[38]. Upon mixing the two partners together, an irreversible covalent isopeptide bond spontaneously forms between the two partners, creating the final rOMV product that can then be purified for use as a vaccine[38]. This innovative approach does not involve the burdensome chemistries associated with the production of conjugate vaccines. In addition, the Spy-Tag system allows the targeting of potentially problematic tumor antigens that require glycosylation to maintain immunogenicity and therefore cannot be synthesized in host non-glycosylating bacteria.

5. Immunogenic properties of OMVs.

In addition to the relative ease with which rOMVs can be engineered to express tumor-associated antigens for use in immunotherapeutic vaccines, such rOMV-based candidate vaccines also offer novel immunogenic properties associated with nanomedicines that cannot be duplicated using conventional parenterally administered subunit vaccines. As previously discussed, intramuscular administration of engineered rOMVs in the size range of 30 – 100 nm are capable of draining into regional lymph nodes and inducing robust activation of antigen presenting cells due to several types of TLR agonists present within the vesicle; larger OMVs can quickly be phagocytosed by peripheral DCs and subsequently trafficked to the lymph node to initiate adaptive immunity.[30] It has also recently been reported that *in situ* delivery of rOMVs exported after oral administration of host bacteria also results in striking immunogenicity and therapeutic efficacy against gastrointestinal tumors.[39] *E. coli* was engineered to express vesicles encoding a targeted protein naturally expressed by MC38 colon tumor cells implanted into C57BL/6 mice. Mice received 10^9 colony forming units (CFUs) of viable host bacteria delivered orally. Interestingly, the tumor antigen was fused in tandem to the Fc fragment of mouse IgG to increase targeting of these novel rOMVs to regional dendritic cells through the DC neonatal Fc receptor. Remarkably, oral administration of *E. coli* delivering rOMVs expressed in the gastrointestinal tract effectively induced tumor-specific immunity against MC38 colon tumor cells that had been implanted subcutaneously, suggesting effective lymphocyte trafficking from the underlying lamina propria to gut-draining lymph nodes. The tumor-specific rOMVs not only inhibited tumor growth but also protected animals against tumor re-challenge. Increased levels of infiltrating CD4+ and CD8+ T cells, as well as DCs and activated neutrophils, were observed in tumor tissue, while levels of immunosuppressive T_{reg}s were inhibited compared to the oral phosphate-buffered saline (PBS) control group. Interestingly, it was also reported that mice receiving orally administered purified rOMVs (versus bacteria delivering rOMVs *in* situ) were not protected against tumor challenge.[39]

6. OMVs and the EPR effect.

While the intramuscular and oral routes of rOMV vaccination appear promising for the treatment of solid tumors, it is the potential administration of rOMVs by the intravenous route that offers more intriguing possibilities for tumor immunotherapy. While still able to circulate into lymph nodes to stimulate antigen presenting cells, systemically administered rOMVs have also been reported in experimental animal models to directly access tumor tissue through leaky blood vessels within the tumor mass through a phenomenon called enhanced permeation and retention (EPR; **Figure 1B**). [28, 40] Some rOMVs given intravenously circulate throughout the body and are cleared by Kupffer cell fixed macrophages of the liver.[41-44] Dendritic cells and macrophages in the spleen also clear circulating rOMVs, with subsequent presentation of processed antigens to naïve T and B cells.[45] For those particles arriving at the tumor, entry of rOMVs into surrounding tumor tissue by EPR occurs predominantly by passive diffusion, while undiffused rOMVs pass through the tumor and back into circulation. Active transport of nanoparticles by specialized cells lining tumor vasculature has also been recently reported[46], but it is unclear how significant this phenomenon is versus the passive diffusion mechanisms of EPR. Although significant antigen-specific immunity can therefore take place through adaptive immune mechanisms elicited in the spleen and lymph nodes following systemic administration, these adaptive responses can be enhanced by innate immune stimulation occurring in the tumor microenvironment through the EPR effect.

The EPR effect was first described by Yasuhiro Matsumura and Hiroshi Maeda in 1986[ref47] and ushered in great hope for targeted tumor therapy using nanoparticles preloaded with chemotherapeutic drugs to specifically destroy solid tumor tissue while avoiding the off-target tissue toxicity typically encountered by systemically administered chemotherapy treatments. Their work clearly demonstrated target-specific accumulation of nanoparticles within solid tumors through the EPR effect and no significant accumulation in otherwise healthy tissues. The EPR-sensitivity of a newly emerging tumor is a direct result of the rapid and

unrestricted proliferation of tumor cells. To maintain this vigorous growth, tumors with a diameter of ~1-2mm in diameter cannot depend on simple diffusion of nutrients and must establish a robust blood supply for continued expansion.[40] To meet these rapidly escalating demands for oxygen and nutrients, tumor vasculature associated with the release of elevated levels of vascular growth and permeability factors (including bradykinin, nitric oxide and prostaglandins) becomes dense and chaotic.[48] These rapidly proliferating blood vessels have deficient basement membranes and fenestrated structures with gaps in the endothelial cells. Pericytes become loosely attached to endothelial cells and smooth muscle cells surrounding these vessels are either deficient or malfunctioning, contributing to locally compromised blood flow.[40, 44, 48]

In addition to the enhanced permeability blood vessels, growing tumors also begin to compromise the lymphatic system which normally functions to drain excess interstitial fluid from tissues back into the circulatory system; this phenomenon is responsible for the "retention" aspect of the **e**nhanced **p**ermeability and **r**etention (EPR) effect. The normal blood pressure created in the circulatory system by the heart creates a hydrostatic pressure that pushes fluid and small molecules out of vessels and into the surrounding tissues; the tight junctions of healthy vessel endothelial cells and attendant pericytes prevent larger macromolecules (and nanoparticles) from passing freely into surrounding tissues. Excess fluid build-up in tissues that could disrupt the required flow of nutrients into tissues is normally prevented by the lymphatic system which efficiently shuttles this fluid back into circulation[42]. This finely balanced fluid recovery system is profoundly disrupted in rapidly growing tumor tissue. The lymph vessels become collapsed due to the rising interstitial pressure generated by rapidly dividing tumor cells pushing up against normal surrounding tissues, as well as a dense highly cross-linked extracellular matrix (ECM) that often surrounds these tumors.[42] This mechanism of compromised intratumoral blood vessels, accompanied by an inefficient lymphatic drainage system and a frequently elevated expression level of inflammatory factors results in the retention of extravasated macromolecules (and nanoparticles) in tumor tissues that defines the EPR effect.[28, 48]

Small tumors show a more uniform EPR effect.[28] Once a tumor has progressed to a diameter greater than 1 mm, cells toward the center of the rapidly growing mass can no longer be supplied by diffusion and can become hypoxic and necrotic in the absence of sufficient blood supply[42] Hypoxic cells release pro-angiogenic factors to stimulate endothelial cells from nearby blood vessels to rapidly divide, but these immature vessels cannot penetrate into the central necrotic region of the tumor.[28, 42] Consequently, with larger solid tumors, the vasculature becomes restricted to the more peripheral regions and the EPR effect becomes significantly diminished and heterogenous across the tumor. However, it is important to note that this peripheral highly vascularized area of a larger tumor is still the most vigorously growing zone of the tumors, and consequently still remains vulnerable to penetration by nanoparticles through the EPR effect.[48]

7. Exploiting the EPR effect to elicit adaptive immunity.

Given that large tumors present only limited access of circulating nanomedicines through the extracellular matrix and into the microenvironment of peripheral tumor tissue, this strongly supports a therapeutic strategy in which small emerging tumors rather than larger solid tumors are targeted by systemic administration of small tumor-specific nanoparticles and rOMVs. Data from experimental murine models indicates that nanomedicines in the size range of 100 – 200 nm in diameter are considered optimal for extravasation into solid tumor tissue by the EPR effect, while avoiding the clearance mechanisms of the liver and spleen.[48] However, significant accumulation in tumors can take up to 24 hours or more[28], suggesting either that nanomedicines must be engineered for long half-lives in circulation or must be given in multiple spaced doses. These observations strongly suggest that parenterally administered tumor-specific rOMVs in the size range of 30 – 100 nm may prove to be very effective in the therapeutic treatment of solid tumors through both access to the tumor microenvironment through the EPR effect, as well as trafficking of activated cDC1s to regional lymph nodes to elicit innate and adaptive tumoricidal CD8+ T cell responses.

Cross presentation of antigens is central to the presentation of tumor antigens by activated DCs to naïve CD8+ T cells to elicit cytotoxic immunity.[49] It has now been directly demonstrated by Schetters et al.[50] that target antigens expressed on the surface of rOMVs can be efficiently cross-presented via the MHC-I pathway to CD8+ T cells to trigger antigen-specific T cell activation. Although not explicitly targeting tumor-associated antigens, this work used human monocyte-derived DCs, murine bone marrow-derived DCs and CD11c+ splenic DCs to provide strong evidence for cross-presentation using model ovalbumin (OVA) antigens expressed on the surface of rOMVs in host bacteria using the bacterial autotransporter Hbp. These investigators showed for

the first time that OMV-associated antigens can be cross-presented by DCs to antigen-specific CD8+ T cells; these recombinant OVA-OMVs induced maturation and cross-presentation by both cDC1 and cDC2 cells, although cDC1s were more efficient at cross-presentation.[50] The ability of rOMVs to elicit robust T cell activation through cross-presentation of antigens was suggested to be linked to the presence of pro-inflammatory TLR agonists including LPS, which trigger the upregulation of co-stimulatory molecules including CD80, CD86 and CD70 needed for efficient T cell activation. The importance of TLR agonists to efficient T cell activation was demonstrated more clearly by Twilhaar et al.[51] using liposome nanoparticles used to stimulate cross-presentation in DCs. They reported that immunization with liposomes re-engineered to incorporate the potent TLR4 adjuvant monophosphoryl-lipid A (MPLA), along with a model synthetic OVA peptide containing both CD4+ and CD8+ epitopes, significantly improved both CD4+ and CD8+ T cell responses compared to unmodified liposomes. Taken together, these observations strongly support the feasibility of employing rOMVs as candidate therapeutic vaccines targeting solid tumor-associated antigens by eliciting broad tumor-specific responses.

8. Pre-clinical efficacy studies in experimental animal models.

Very promising results from pre-clinical studies in murine tumor challenge models point to success using OMVs to elicit a tumoricidal EPR-effect against solid tumors in the absence of co-delivered tumor antigens, as well as the ability to elicit tumor-specific cytotoxic responses against surface-expressed antigens. Kim at al.[52] tested engineered OMVs from non-pathogenic Gram-negative *E. coli* W3110 with reduced endotoxicity through modification of the lipid A moiety of LPS by deletion of *msbB*. MsbB is an acyltransferase that transforms penta-acylated lipid A into the more reactogenic hexa-acylated form that is a strong TLR4 agonist[53]; therefore deletion of *msbB* was not only intended to reduce TLR4-mediated inflammatory responses but was also intended to extend the half-life of vesicles in circulation. These vesicles were systemically administered in four 5μg unadjuvanted doses spaced 3 days apart, and given to 6 week old BALB/c or C57BL/6 mice bearing syngeneic subcutaneous tumors from CT26 or MC38 colon tumor cells respectively[52]. They did not specifically target tumor-associated antigenic targets in this study, instead relying only on disruption of the immunosuppressive tumor microenvironment to achieve tumor clearance. Remarkably, Δ*msbB* OMVs passively accumulated **only** in tumor tissue, an effect also observed with OMVs isolated from Gram-positive *Staphylococcus aureus* and *Lactobacillus acidophilus*. Accumulated vesicles induced IFN-γ responses associated with NK and T cells and elicited full eradication of established tumors with no notable side-effects; interestingly, subcutaneous administration of the host bacteria from which the vesicles were isolated could not induce an anti-tumor response[52]. One important caveat to this very encouraging study is that the tumors systemically treated with OMVs were small, with diameters of <1mm.

As previously mentioned, the EPR effect is significantly influenced by the half-life of circulating vesicles in the blood stream that are subject not only to clearance in the liver and spleen, but also to attack by complement and other innate defense mechanisms in the blood. To improve vesicle half-life and reduce any potential for a cytokine storm triggered by intravenous administration of unmodified OMVs, Qing et al.[54] investigated the use of vesicles coated with dissolvable calcium phosphate to temporarily shield vesicles from phagocytic cells until deposition by extravasation into tumors. Similar to the Kim experiment, vesicles purified from *E. coli* were administered in five 5μg doses of unadjuvanted vesicles spaced 2 days apart. Importantly, larger tumors with an initial diameter of 5 mm were targeted in these experiments, but again they did not specifically target tumor antigens. As expected, systemically administered coated vesicles were not cleared rapidly from the bloodstream, did not engender OMV-specific serum IgG responses, and did not induce splenomegaly. Rather, circulating vesicles were reported to accumulate in solid CT26-induced tumor tissue in BALB/c mice, accompanied by a reversal of M1/M2 polarization away from anergic M2 and towards activated M1 macrophages. A significant increase in infiltrating effector CD8+ T cells, a reduction in immunosuppressive T_{reg}s, an increase in tumor apoptotic cells, and higher overall survival rates were also reported[54].

In a further advancement of this OMV therapeutic strategy, Cheng et al.[55] investigated the therapeutic efficacy of rOMVs engineered in *E. coli* to target tumor specific antigens in a syngeneic C57BL/6 MC38 colon cancer challenge model. This group used vesicles formed by over-expression of the hemolysin ClyA and attached tumor-specific antigens to the outer surface of vesicles using the Plug-and-Display SpyCatcher technology.[38, 56] They reported impressive efficacy in mice when targeting an irrelevant OVA peptide transgenically expressed in the MC38 cells; they also

targeted the MC38-specific neoantigen Adpgk in separate experiments, again with impressive anti-tumor responses. Interestingly, tumor eradication was accomplished in mice immunized subcutaneously with three 50µg doses of purified unadjuvanted vesicles spaced 4 days apart and initiated 3 days after implantation of tumor cells; presumably drainage of vesicles into local lymph nodes would be the primary immunological mechanism involved in eliciting tumor-specific immunity. Targeted vaccination against the Adkhp neoantigen led to complete regression of tumors in 60% of mice, with CD4+ and CD8+ T cells, activated neutrophils, and DCs all significantly elevated in MC38 tumor tissues after subcutaneous immunization, but with reduced levels of immunosuppressive T_{reg}s. [55]

Taken together, these data strongly suggest that OMVs can achieve positive therapeutic results against solid tumors in three ways 1] passive targeting of tumor tissue through the EPR effect to disrupt the immunosuppressive environment, 2] enhanced activation of myeloid and antigen presenting cells through surface displayed endogenous TLR agonists, and 3] eliciting critical adaptive immunity against targeted tumor associated antigens that are surface exposed, properly folded, and stably expressed in properly engineered vesicles. However, the efficacy of presumably optimized rOMVs will still depend on the size of the tumor against which vesicles must induce immunity. As discussed above, the larger the tumor, the higher the outward interstitial pressure pushing against collapsing blood and lymphatic vessels, and the lower the efficiency of EPR-based extravasation of rOMVs out of blood vessels into the tumor microenvironment, as well as out of the tumor into regional lymph nodes. This potentially significant limitation to rOMV-based therapeutic treatment has recently been indirectly addressed by Islam et al.[57] who investigated the efficiency of the EPR effect in extremely large tumors and the effect of using vasodilators to increase blood flow and improve access of chemotherapeutic nanomedicines. Six-week-old BALB/c mice were subcutaneously implanted with CT26 colon cancer cells and tumors allowed to progress to a starting diameter of 15-18 mm, after which intravenous administration of vasodilators and therapeutics were dispensed. They reported that chemotherapeutic drug delivery increased significantly only in tumor tissue; in other normal tissues, no significant drug accumulation was seen. Therefore, restoration of blood flow by using EPR-effect enhancers improved EPR effect-based drug delivery to these tumors.

9. Animal models and clinical trials.

While the highly promising observations coming from preclinical experimental animal models suggest potential success with tumor-specific rOMV-based immunotherapeutics in future clinical trials, concerns over the failure of other nanomedicines in clinical trials has dampen enthusiasm in this emerging field of cancer therapeutics, at least at the level of passive deposition of vesicles by the EPR effect into tumors to disrupt the immunosuppressive microenvironment.[28, 40, 42, 48, 58] The apparent failure of the EPR effect in clinical settings versus successes reported in experimental animal models may involve several important factors. Immunotherapy experiments carried out in murine tumor challenge models typically involve treatment of relatively small tumors that would be expected to possess high vascularization, active angiogenesis to match tumor growth rate, unrestricted blood flow, and much lower interstitial tissue pressures[28], thereby favoring more efficient extravasation of nanomedicines into the tumor microenvironment. However, treatment of tumors in clinical settings often involves much larger tumors with inadequate vascularization, necrotic regions in the core, and large interstitial pressures that are not adequately modeled in mouse studies.[28] Indeed, Hiroshi Maeda (who was the first to describe the EPR effect) has correctly pointed out that animal research ethics committees at most institutions restrict the use of large tumors (more than 5000 mm^3), possessing the occluded or embolized tumor blood vessels more representative of clinical situations, that could be used more effectively to optimize the EPR effect and therapeutic treatments based on the EPR effect.[59] This implies that to more accurately optimize outcomes in clinical trials based on testing in experimental animal models, immunotherapeutic therapies must be initiated in patients when tumors are small.

Murine tumors are also much larger in relation to total body mass than what is typically encountered in humans. This would suggest that the probability of systemically administered nanomedicines reaching animal tumors would be much higher than for nanomedicines circulating in the much larger human body in which the targeted tumor constitutes a much smaller percentage of total tissue mass exposed to the circulation.[40, 42, 58] In addition, the rate of blood flow in mice is slower than that observed in humans.[48] For example, it has been reported that the flow rate for a normal mouse liver and muscle tissue is approximately 1.8 mL/100 g/min and 0.91 mL/100 g/mL respectively; the analogous flow rate in normal human liver and muscle tissue is 1450mL/100g/mL and 750mL/100g/mL respectively.[60] The higher

blood flow in humans will therefore create higher shear forces and less time for extravasation into tumor tissues than what can occur in mouse tumors even if tumors are small.

Another important parameter to consider in experimental tumor challenge models is the rate of tumor growth and host lifespan.[42,48] Rapidly growing tumor tissues in animal models induce very rapid angiogenesis leading to disorganized vascular walls punctuated with leaky fenestrations. Such tumors are typically induced in young mice and develop within days to weeks (depending on the size to be experimentally targeted for therapy), while in humans, tumors generally develop and progress over years.[40] Consequently, smaller tumors in mice are relatively homogeneous in tumor tissue phenotypes and between individual animals, whereas clinical tumors that have taken years to develop under at least some level of immune surveillance display much more heterogeneity in size, microenvironment, and vessel leakiness between patients and within individual tumors.[58]

While several of these limitations of the EPR effect in clinical trials can be addressed simply by targeting therapy to small tumors in which the EPR effect would be expected to function more efficiently, other concerns must also be addressed regarding the potential reactogenicity of rOMVs administered intravenously to enable EPR-based deposition. However, the introduction of bacterial OMVs into the bloodstream does not *necessarily* lead to a potentially unacceptable inflammatory response in humans. It was recently reported by Tulkens et al.[61, 62] that up to 10^6 LPS-positive bacterial vesicles per milliliter of blood plasma were detected in non-septic cancer patients after having undergone chemotherapeutic or radiation treatments. The presence of these circulating OMVs was associated with increased permeability of the intestinal epithelial barrier and was hypothesized to be related to disruption of enterocyte tight junctions during treatment, leading to vesicles from resident flora seeding the bloodstream. In addition, studies by S.A. Rosenberg et al.[63, 64] reported that intravenous infusion of a genetically modified *Salmonella* strain expressing penta-acylated lipid A (as described in the Kim experiments[52] with purified OMVs) into the blood of metastatic cancer patients also did not result in unacceptable inflammatory reactions, even after 4 hours of continuous infusion of these attenuated *Salmonella* strains at the maximum tolerated dose 3×10^8 CFU/m^2 (CFU; colony forming units); interestingly, bacteria quickly disappeared from circulation within 2 hours of cessation of the infusion. However, to improve the clinical acceptability of rOMVs administered parenterally, it will undoubtedly be prudent to reduce potential reactogenicity to satisfy Food and Drug Administration (FDA) requirements ensuring the safety of human vaccine candidates. Possible solutions may include the genetic manipulation of rOMVs to minimize reactogenicity. Given that the synthesis of biologically active LPS is accomplished through the action of multiple enzymes catalyzing the phosphorylation and acylation of lipid A [ref 65], the genetic deletion of several of these enzymes can result in LPS that retains its functional characteristics in the outer membrane but is diminished in its ability to activate TLR4. However, this is a double-edged sword because activation of DCs through TLRs is a potent pathway for initiating tumor-specific innate immunity and manipulation of TLR activity therefore requires optimization to achieve limited inflammatory responses while still retaining the ability of these modified OMVs to tip the balance towards induction of adaptive immunity.

10. An example of rOMV versatility.

In an attempt to further advance the use of rOMVs in therapeutic cancer vaccines, we have recently developed an innovative rOMV-based immunotherapeutic against colorectal cancer that seeks to take advantage of genetically engineered vesicles with reduced TLR activity to control unacceptably high inflammatory responses while still maintaining robust tumor-specific adaptive immunity. These vesicles carry modified lipid A moieties within the lipopolysaccharide of the outer membrane that display significantly reduced TLR4 activity *in vitro*. The details of this novel rOMV-based expression system will be published in detail elsewhere and will only be briefly described here to provide another example of the versatility of rOMVs as potential immunotherapeutic treatments against cancer.

To create our rOMV-based system, we engineered an osmotically controlled synthetic expression cassette encoding the enzyme PagL into an attenuated strain of *Salmonella*. It was recently reported by Elhenawy et al.[66] that over-expression of PagL in *Salmonella* induces elevated production of OMVs; while Gram-negative bacteria are normally capable of exporting OMVs, over-expression of PagL dramatically increased the level of OMV export. It therefore becomes possible to further engineer into such strains the ability to express additional target tumor antigens that when expressed on the surface of the bacterium in the context of additional PagL co-expression, leads to export of rOMVs that can now be purified for use as therapeutic cancer vaccines. For this particular application, we chose to express domains from two antigens over-expressed in colorectal cancer

called carcinoembryonic antigen (CEA) and mucin 1 (MUC1). CEA is a ~180 kDa surface glycoprotein expressed on various tumor tissues including colorectal cancer and is hypothesized to have both cell adhesion and pro-angiogenic properties[67, 68]; over-expression has been reported to result in hypoglycosylation, potentially exposing epitopes normally masked in healthy tissue by glycosylation. The other targeted antigen, MUC1, is normally present on most polarized mucosal epithelial tissue in a heavily glycosylated form. However, when over-expressed in solid tumor tissue, this antigen can also become significantly under-glycosylated[69, 70], which as with over-expression of CEA, opens up normally masked epitopes to immune surveillance[70, 71]. Our novel tumor antigen expression cassette was engineered as a protein fusion of the CEA and MUC1 domains, positioned downstream from the gene cassette encoding PagL; therefore, osmotic induction of this synthetic operon resulted not only in high levels of PagL but also of the CEA-MUC1 protein fusion exported to the surface of exported rOMV vesicles.

Remarkably, PagL is a lipid A deacylase that reduces the number of acyl groups from the highly reactogenic hexa-acylated species present in *Salmonella* to a less reactogenic penta-acylated species.[72] Therefore, over-expression of PagL not only catalyzes the efficient formation of high levels of exported rOMVs that can be purified and used as immunotherapeutic vaccines, but also simultaneously reduces the potential reactogenicity of these vesicles to improve clinical acceptability. As it has been observed that vesicles purified from *Salmonella* are frequently contaminated with flagella (a very powerful TLR5 agonist), we elected to remove the gene encoding flagellin and replace it with *lpxE* from *Francisella novicida*. LpxE is a lipid A 1-phosphatase which dephosphorylates lipid A to produce a less reactogenic monophosphoryl species. Therefore, the combination of PagL activity and LpxE activity produces a lipid A moiety within our rOMVs that is potentially even less reactogenic than the structurally similar and clinically approved adjuvant MPLA.[73, 74]

Despite the reduction in TLR4 and TLR5 activity, these modified vesicles still maintained robust immunogenicity when tested in C57BL/6 mice, with excellent serum IgG responses against our CEA-MUC1 fusion protein, as well as excellent antigen-specific cellular responses as judged by INF–γ enzyme-linked immunosorbent spot (ELISPOT) assays. Encouraged by these very strong humoral and cellular responses, we conducted an initial efficacy study using a syngeneic C57BL/6 mouse model subcutaneously implanted with MC38 murine tumor cells expressing either CEA or MUC1. Mice were implanted subcutaneously with 300,000 tumor cells on day 1 and then treated intravenously with 0.75 micrograms of vesicles (based on quantitation of LPS concentration using a KDO assay) on days 3, 5, 7, and 9. Progression of tumors was monitored by calculating tumor volumes through day 28. As shown in **Figure 2** for mice challenged with MC38-MUC1, we observed a 100% reduction in morbidity in mice treated with rOMVs; mice receiving rOMVs deleted for flagella only (i.e. rOMV$^{\Delta fliC}$not expressing LpxE) still experienced some progression of tumor size, but for mice receiving rOMVs expressing LpxE (i.e. rOMV^{lpxE} deleted for flagellin expression and expressing LpxE), we observed up to 50% total remission of tumors in these mice. It is clear from these preliminary observations that bacterial rOMVs can be engineered to efficiently display tumor antigens in the context of membrane-bound LPS with reduced TLR-mediated stimulation of innate immunity but can still retain excellent immunogenicity and efficacy in experimental tumor challenge models.

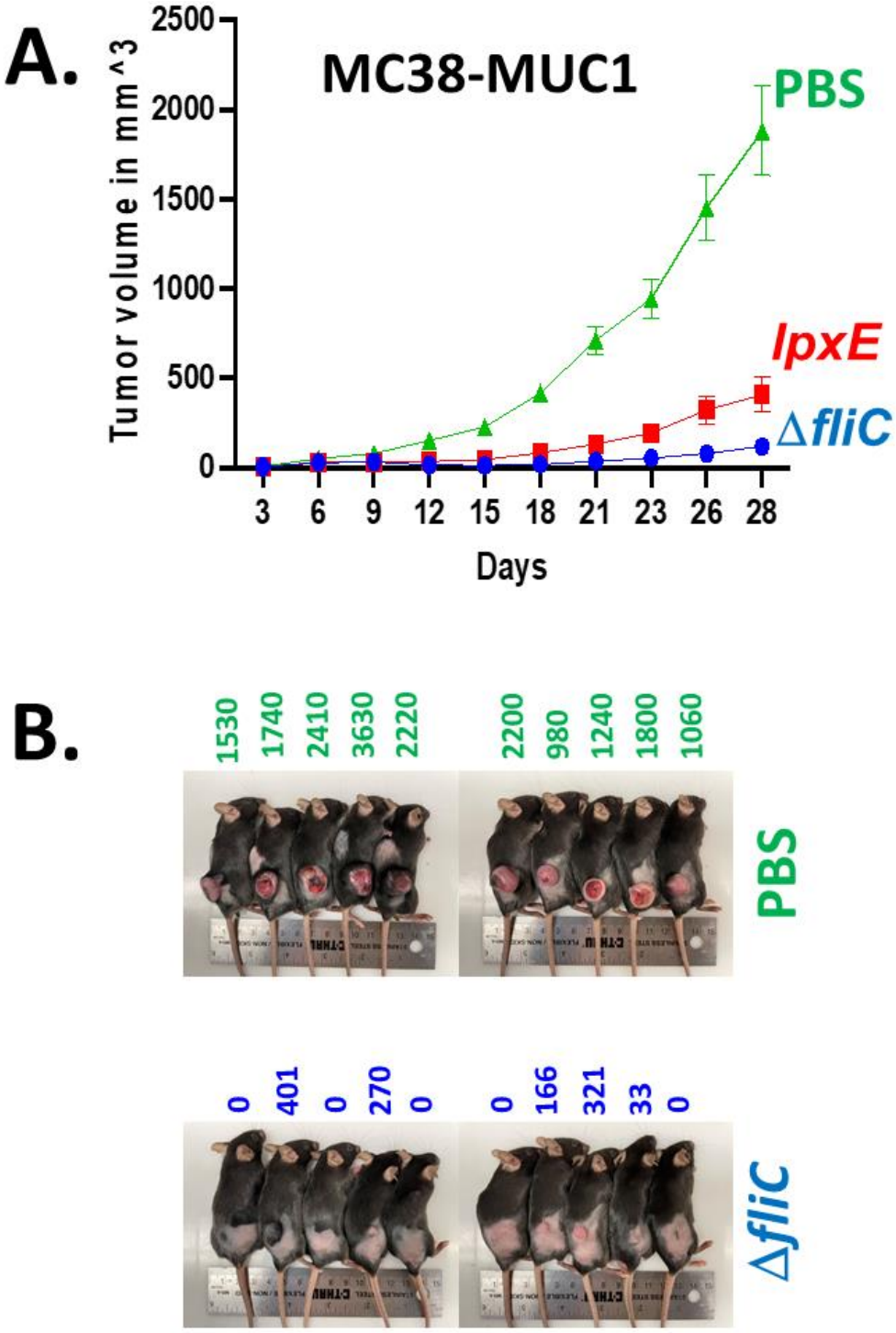

Figure 2. Immunotherapeutic treatment of C57BL/6 mice (10 mice per group) implanted with 300,000 MC38 colon cancer cells engineered for constitutive expression of the MUC1 antigen. Cells were implanted subcutaneously on day 0; mice were then treated on days 3, 5, 7, and 9 with intravenous injections of either with PBS or 0.75 µg per dose of either Δ*fliC* or *lpxE* rOMVs expressing a CEA-MUC1 targeted tumor-associated fusion protein. Progression of tumors as measured by tumor volume are shown in panel A; data plotted as mean volumes and standard error of the means represented by bars. Statistical significance was evaluated by ANOVA with Geisser-Greenhouse correction and a Tukey multiple comparisons analysis; Δ*fliC* vs. *lpxE*, $p = 0.05$; Δ*fliC* vs. PBS, $p = 0.0001$; lpxE vs. PBS, $p=0.0004$. Experimental animals are shown in panel B at the conclusion of the experiment on day 28; values for tumor volumes are indicated.

CONCLUSIONS

Bacterial outer membrane vesicles constitute a remarkably flexible platform for the immunotherapeutic treatment of cancer. rOMVs specifically engineered to target tumor-associated antigens are endowed with several unique properties that can enhance tumor clearance: 1] passive deposition into nascent solid tumors of intravenously administered rOMVs through extravasation out of leaky nascent tumor vasculature to disrupt immunosuppression and activate innate immunity, 2] the capacity to induce cancer antigen-specific adaptive immunity through delivery of tumor associated target antigens to regional lymph nodes via either draining rOMV vesicles or trafficking of rOMV-activated dendritic cells, and 3] versatility as a platform-based technology to efficiently incorporate newly identified tumor-associated antigens into modified rOMVs to quickly create the next iteration of vaccine candidates. Any potential reactogenicity associated with rOMVs can be minimized either by re-engineering of potentially reactive bacterial outer membrane proteins, lipoproteins, and lipopolysaccharides using state-of-the-art molecular biology techniques, or by testing alternate routes of administration including parenteral or oral routes. Although clinical trials have yet to be completed for examining the safety and efficacy of rOMV-based vaccines against solid tumors, it is becoming increasingly important for clinical success that patients be screened for the antigens specifically targeted by rOMVs (or any other tumor-specific therapeutic vaccine, for that matter), and to also begin treating patients before tumors progress to late-stage necrotic cancers that are difficult to treat under any circumstances. In our view, rOMVs represent an untapped technology that could significantly advance the field of immunotherapeutic treatments against tumors. The vast arsenal of powerful molecular biology tools now available to investigators will undoubtedly be applied more creatively in the future to solve any remaining safety or production issues that would otherwise impede the further advancement of this promising vaccination strategy.

CONFLICTS of INTEREST STATEMENT

Although this work was sponsored by Irazu Bio, the authors of this work are not employees of Irazu Bio, nor do they have any financial interests in Irazu Bio. Therefore, the authors have no conflicts of interest to declare.

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