Adjuvant-Loaded Subcellular Vesicles Derived From Disrupted Cancer Cells for Cancer Vaccination

Alexander S. Cheung, Sandeep T. Koshy, Alexander G. Stafford, Maartje M. C. Bastings, and David J. Mooney*

Targeted subunit vaccines for cancer immunotherapy do not capture tumor antigenic complexity, and approaches employing tumor lysate are often limited by inefficient antigen uptake and presentation, and low immunogenicity. Here, whole cancer cells are processed to generate antigen-rich, membrane-enclosed subcellular particles, termed “reduced cancer cells”, that reflect the diversity and breadth of the parent cancer cell antigen repertoire, and can be loaded with disparate adjuvant payloads. These vesicular particles enhance the uptake of the adjuvant payload, and potentiate the activation of primary dendritic cells in vitro. Similarly, reduced cancer cell-associated antigens are more efficiently presented by primary dendritic cells in vitro than their soluble counterparts or lysate control. In mice, vaccination using adjuvant-loaded reduced cancer cells facilitates the induction of antigen-specific cellular and humoral immune responses. Taken together, these observations demonstrate that adjuvant-loaded reduced cancer cells could be utilized in cancer vaccines as an alternative to lysate.

1. Introduction
Therapeutic cancer vaccines immunize the patient against cancer antigens in order to generate immune effector cells that can recognize and eliminate cancer cells. Cancer vaccination is challenging for several reasons. First, tumors are heterogeneous populations with high degrees of both inter- and intra-tumoral antigen variability.\(^1\) Second, tumors possess tolerogenic mechanisms that enable them to avoid immune destruction.\(^2\) Third, tumors are dynamic populations that can evolve in response to weak selective pressures induced by suboptimal vaccination.\(^3\) In order to overcome these challenges, it is important for a cancer vaccine to elicit a response that is personalized to the patient’s unique cancer antigen repertoire, and broad enough to capture the antigenic diversity of the tumor. The vaccine must also facilitate efficient antigen presentation and promote robust cytotoxic effector cell function in order to overcome the immunosuppressive mechanisms imposed by the tumor.

In general, two approaches exist to cancer vaccination: 1) targeted approaches, which immunize against defined tumor antigens and 2) broad vaccination against a breadth of undefined tumor antigens. Targeted approaches, such as subunit vaccines, are attractive because they generally
target antigenic determinants that are known to be uniquely overexpressed and immunogenic. However, because targeted vaccines do not reflect the antigenic complexity of the tumor, suboptimal vaccinations that fail to induce efficient antigen spread can potentially facilitate the selection of lowly immunogenic subpopulations, leading to immune escape.\textsuperscript{[3]} Additionally, targeted approaches are commonly non-personalized in that they immunize against prototypic cancer antigens, which precludes the use of such approaches for a large patient population that either has a cancer for which a characteristic target has not been identified, or is refractory for this particular known target. Alternatively, strategies that utilize material derived from the tumor as an antigen source vaccinate against a breadth of prospective tumor antigens in a patient-specific manner. The current standard for such primary antigen preparations is tumor lysate, the soluble fraction obtained after freeze-thawing cancer cells. Lysate-based approaches have the advantage of capturing both the uniqueness and breadth of the tumor antigen repertoire, without the need for pre-knowledge of specific antigen targets. This is particularly advantageous in cancer types with high mutational loads (e.g., melanoma), in which patients frequently have unique and extensive mutanomes encoding neoantigens not subject to central tolerance, which could potentially facilitate efficient vaccination.\textsuperscript{[4,5]} Despite these advantages, tumor lysate possesses limited immunogenicity, and the soluble lysate constituents are not efficiently taken up by antigen-presenting cells, limiting the immune response generated.\textsuperscript{[6]}

Previous work has demonstrated that the physical association of the antigen with a molecular danger signal, such as a Toll-like receptor (TLR) agonist, for example through chemical conjugation\textsuperscript{[7,8]} or co-loading onto a nano- or microscale colloidal scaffold\textsuperscript{[9-12]} significantly increases its presentation efficiency, even when compared to the same amount of soluble antigen and danger cue admixed but not physically associated. Indeed, the presence of danger signal and antigen in the same phagosomal compartment has been shown to be important for the efficient presentation of that antigen.\textsuperscript{[13,14]} It has also been shown that compared to a soluble counterpart, antigens associated with a “phagocytic substrate”, such as an engineered colloid,\textsuperscript{[9,10,15]} or cell debris,\textsuperscript{[16]} or that is packaged into apoptotic blebs,\textsuperscript{[17]} or tumor exosomes,\textsuperscript{[18]} are more efficiently expressed by antigen-presenting cells. The application of nano- or microscale materials as delivery vehicles in cancer vaccines has allowed for the development of many promising targeted vaccines, but non-targeted approaches based on such platforms are much more limited. This is largely owing to the difficulty of loading complex undefined mixtures of proteins into such systems, while maintaining a reasonable degree of colloidal stability. In addition, the use of exogenous materials as colloidal delivery vehicles can potentially lead to challenges associated with scale-up and reproducibility, as well as regulatory hurdles to translation.

In light of these observations, the goal of this work was to develop an alternative method for processing cancer cells in which the antigen diversity of the parent cells is maintained but the antigen content is reformulated to facilitate efficient antigen presentation. To this end, we hypothesized that whole cancer cells could be broken down into membrane-enclosed vesicular compartments and loaded with adjuvant. The benefits of such a system are: 1) the unique antigen repertoire of the parent cells is reflected, 2) the breadth of antigenic diversity of the parent cell population is captured, 3) associated cancer antigens are presented in a highly immunogenic format, in contrast to lysate, the current standard, and 4) endogenous cell material functions as the delivery vehicle, obviating the input of exogenous carrier material. In this work, we investigated two approaches to mechanically disrupt whole cancer cells, extrusion and sonication, in order to generate vesicular compartments we termed “reduced cancer cells” (RCCs). We demonstrate that in contrast to extruded RCCs, sonicated RCCs retain protein from diverse intracellular compartments with a protein distribution that is highly representative of the parent whole cells, and leverage this property for vaccine design. We show that sonicated RCCs can be loaded with diverse payloads, and that adjuvant-loaded RCCs are highly immunogenic and facilitate more efficient antigen presentation in vitro than cell lysate or purified soluble antigen. When administered as a vaccine in vivo, RCCs can also be used to stimulate antigen-specific cellular and humoral immune responses. Overall, this work describes an approach for reformulating whole cancer cells into immunogenic, antigen-rich particles that may represent a superior alternative to the derivation of lysate.

2. Results

2.1. Derivation and Characterization of RCCs

Whole B16-F10 melanoma cells were disrupted via either mechanical extrusion (eRCCs) or sonication (sRCCs) to generate RCCs that were subsequently purified using density centrifugation. Both methods generated RCCs with nano- to microscale size distributions (Figure 1A). Dynamic light scattering (DLS) measurements indicated that eRCCs had an average hydrodynamic diameter of around 400 nm whereas sRCCs had an average hydrodynamic diameter of around 500 nm associated with a broader, less monodisperse distribution and a higher PDI (Figure 1A, and Figure S1A,B in the Supporting Information). Both eRCCs and sRCCs had comparable zeta potentials of about −30 mV (Figure 1B, Figure S1C, Supporting Information), consistent with what has been reported previously for purified mammalian cell membranes.\textsuperscript{[16,20]} Examination of the RCCs using transmission electron microscopy (TEM) revealed structures within the size range predicted by DLS. Also consistent with the DLS measurements, the TEM images showed that the eRCC structures appeared generally more monodisperse whereas the sRCC structures varied more in size (Figure 1C,D, Figure S1D,E, Supporting Information). Enhancement of contrast upon negative staining with uranyl formate suggests that both eRCCs and sRCCs represent enclosed structures as would be expected of membrane-bound vesicles. Although partial membrane collapse of the RCCs was observed following staining and dehydrating during TEM sample preparation, it is likely that the RCCs are spherical structures in the hydrated state. These observations suggest that by disrupting
whole cancer cells via either mechanical extrusion or sonication, membrane-enclosed vesicular particles are obtained. Importantly, these particles may retain endogenous cell membrane-associated proteins in the membranous “shell” of the particles, as well as soluble intracellular proteins in the aqueous “core”.

In order to investigate the prospective antigen content of eRCCs and sRCCs, total and specific RCC protein retention was characterized. eRCCs were found to retain 10.8 ± 0.7% of the total parent cell protein content whereas sRCCs were found to retain 22.7 ± 3.4% (Figure 2A). Qualitative analysis of total protein content using Coomassie staining showed that eRCCs exhibited a depletion of a number of prominent bands observed in the parent whole cell lysate whereas sRCCs maintained a protein distribution qualitatively more similar to the parent whole cell lysate (Figure 2B). When the same amount of total protein was loaded, Western blotting for proteins localized to various cellular compartments indicated that while eRCCs showed a marked enrichment of membrane-associated proteins with a concomitant loss of proteins in other compartments, notably cytoplasmic and nuclear, protein retained by sRCCs was highly representative of all compartments of the parent cells based on the markers evaluated (Figure 2C). Given that many tumor antigens are localized to the cytoplasm and the nucleus,[21] the ability to retain these fractions is likely to be functionally advantageous for a cancer vaccine. Because of this, as well as their superior gross protein retention, sRCCs were used for all subsequent analyses. For all functional assays, sRCCs were typically prepared and used fresh. Although sRCCs were observed to settle over time, particles suspended in phosphate-buffered saline (PBS) could be stored for several days at 4 °C and redispersed with brief bath sonication without a significant change in the average particle size (Figure S2A, Supporting Information). Because the RCCs are comprised entirely of cell material, it is expected that they can be completely degraded when taken up by phagocytic cells, such as antigen-presenting cells (APCs). In contrast, we observed that in the absence of cells, the RCCs were not significantly degraded in the presence of serum for several days, although serum-mediated aggregation was evident over this time (Figure S2B, Supporting Information).

2.2. RCCs as Adjuvant Delivery Vehicles

To evaluate whether sRCCs could be loaded with adjuvant, a 5′-fluorophore-conjugated CpG DNA was used as a model adjuvant, and loaded into the sRCCs by mixing the parent cells with CpG and sonicating the CpG/cell suspension. CpG is a hydrophilic oligodeoxynucleotide-based adjuvant and a ligand for the endosomally localized TLR9, an immune-activating pattern recognition receptor (PRR). CpG has been shown to be an effective adjuvant in cancer vaccine formulations.[22–26] When sRCCs were stained with a membrane dye and subjected to density centrifugation in a two-step (10% /50%) iodixanol gradient, significant enrichment of the dye was observed at the interface of the steps, representing
the membrane-enclosed sRCC-rich fraction (Figure 3A). While free fluorescent CpG did not localize to a specific fraction upon density centrifugation, when CpG was mixed with the cell suspension and sRCCs were derived from the CpG/cell mixture, a fraction of the CpG was found to colocalize with the interfacial RCC band following density centrifugation (Figure 3A,B), suggesting CpG loading of the resultant sRCCs. Confocal microscopy of sRCCs also revealed colocalization of a membrane-specific dye and the fluorescent CpG (Figure 3C). To preclude the possibility that the fluorescent tag was facilitating loading, sRCCs were also loaded with untagged CpG and loading was quantified using high-performance liquid chromatography (HPLC) and measuring the absorbance at 260 nm. Loading of fluorescent CpG was similarly evaluated using HPLC and measuring the absorbance at 260 nm as well as the fluorescence at 488 nm. Comparison of tagged and untagged CpG did not reveal any differences in loading (Figure S3, Supporting Information). Furthermore, no change in elution time was observed when HPLC was used to compare the fluorescent CpG stock and fluorescent CpG that had been sRCC-loaded and recovered following RCC lysing, suggesting that intact CpG is loaded into the RCCs (Figure S4, Supporting Information). CpG loading of sRCCs was evaluated as a function of cell input, and 5e6 cells mL\(^{-1}\) was found to be the optimal cell concentration among those tested (Figure S5, Supporting Information). This cell concentration was used for subsequent experiments involving CpG-loaded sRCCs. Based on such analyses, it was found that CpG could be loaded into sRCCs at 36.4% efficiency, the lipophilic adjuvant monophosphoryl lipid A (MPLA) could be loaded at 1.8% efficiency, and the model antigen ovalbumin (OVA) could be loaded at 7.0% efficiency (Figure 3D).

The ability of RCCs to facilitate the uptake of associated adjuvants by antigen-presenting cells (APCs) was evaluated next using fluorescent CpG as a model adjuvant and primary bone marrow-derived dendritic cells (DCs) as a model APC. CpG associated with RCCs was taken up by DCs with faster kinetics and at a higher magnitude than a comparable amount of free CpG, with around 10% more DCs being CpG+ after 4 hours and beyond, and a more than 4 and 5-fold higher mean fluorescence intensity (MFI) being observed among CpG+ cells at 4 and 8 h, respectively (Figure 4A,B). These observations were confirmed with confocal microscopy wherein significantly more CpG signal was visible in DCs treated with RCC-associated versus free CpG at all time-points evaluated (Figure 4C, Figure S6A, Supporting Information). Taking an orthogonal slice through a CpG+ DC, CpG fluorescence was apparent in the interior of the cell (Figure 4D) demonstrating true internalization of the CpG. This was further confirmed by visualizing slices through the z-axis of CpG+ DCs as CpG-associated fluorescence was observed throughout the cell volume (Figure S6B, Supporting Information). CpG was found to be localized within...
the endolysosomal compartment of DCs after treatment for 4 h (Figure 4E), which is likely to have functional implications as TLR9 is localized in the endosomal compartment. Taken together, these data show faster uptake kinetics and a higher amount of CpG internalization with RCC-associated CpG compared to free CpG, demonstrating a particle-mediated enhancement of payload uptake by DCs.

RCC sidedness, the orientation of the RCC membranes, could potentially have functional implications, and so was also analyzed. In particular, CD47 is a cell surface transmembrane protein that inhibits phagocytosis, and its upregulation is a common mechanism of immune evasion by tumor cells.\cite{27} Immunoelectron microscopy of sRCCs was performed using antibodies that target either the extracellular or intracellular portion of CD47 (Figure 4F). sRCCs were stained by both antibodies suggesting that the plasma membrane-derived structures were likely present in a mixture of right-side out and inside-out orientations. This contrasts with previous reports that plasma-membrane vesicles derived via mechanical extrusion are primarily maintained in a right-side out orientation,\cite{28,29} which could potentially hinder uptake by APCs if functional CD47 is retained.

2.3. Immunologic Functionality of Adjuvant-Loaded RCCs In Vitro

To evaluate the functionality of RCC-associated adjuvants, DCs were treated with CpG and MPLA in either free or RCC-associated forms, and assayed for activation based on upregulation of surface activation markers and production of the proinflammatory cytokine IL-12. Treatment of DCs with blank sRCCs did not promote any increase in surface activation marker expression or IL-12 production compared to mock treatment. In contrast, treatment with sRCCs loaded with either CpG or MPLA significantly upregulated the surface expression of MHC II and the costimulatory molecules CD86 and CD40 on DCs, to a similar or greater degree than the respective free adjuvant (Figure 5A). Notably, treatment of DCs with sRCC-associated adjuvants promoted the significantly greater production of IL-12 than did the same amount of the respective free adjuvants, with a 36% and 80% increase observed for CpG and MPLA, respectively (Figure 5B). Treatment of DCs with CpG-loaded sRCCs also promoted a change in cell morphology characterized by increased spreading and aspect ratio relative to unactivated DCs (Figure S7, Supporting Information). These morphological changes are consistent with what has been previously reported to take place upon in vitro DC activation.\cite{30}

Next, the ability of the RCCs to facilitate antigen presentation by APCs was evaluated using the model antigen OVA. To evaluate the presentation of RCC-associated antigens on MHC II, DCs were treated with sRCCs derived from either wild-type B16-F10 cells (B16-WT), or B16-F10 cells transduced to express a membrane-bound form of OVA (B16-mOVA) (Figure S8, Supporting Information), and co-cultured with MF2.2D hybridoma T cells. MF2.2D cells recognize an OVA-derived peptide presented on MHC II, and produce IL-2 in response to T cell receptor (TCR) stimulation. Whereas no increase in IL-2 production was observed compared to control conditions when the DCs were treated with B16-WT sRCCs, a dose-dependent increase in
IL-2 production was observed with B16-mOVA sRCC treatment over the concentration range tested (Figure 6A).

To evaluate MHC I-mediated cross-presentation of RCC-associated antigens, a process critical for robust anti-tumor immunity, DCs treated with various stimuli were co-cultured with carboxyfluorescein succinimidyl ester (CFSE)-stained primary CD8+ T cells derived from OT-I mice, which express a transgenic T cell receptor specific for an OVA peptide presented on MHC I. T cell activation was evaluated by measuring CFSE dilution as a proxy for T cell proliferation.

First, DCs were treated with either B16-WT sRCCs, B16-mOVA sRCCs, or cancer cell lysate derived from the same number of starting B16-mOVA parent cells. The sRCC samples were either unloaded, or loaded with CpG, and the lysate samples were either used as-prepared, or admixed with the same amount of soluble CpG. Whereas DCs treated with B16-WT sRCCs or B16-mOVA lysate, with or without CpG, facilitated minimal OT-I CD8+ T cell proliferation, DCs...
treated with B16-mOVA sRCCs loaded with CpG facilitated extremely robust OT-I CD8+ T cell proliferation (Figure 6B).

Because it is possible that the difference in antigen presentation efficiency between lysate and RCC could be due to differences in amount of relevant protein being retained by the two derivation procedures, the antigen presentation efficiency of soluble OVA protein was compared to the same amount of OVA protein loaded into B16-WT sRCCs. Consistent with the observations made with endogenously expressed OVA, it was observed that whereas DCs treated with soluble OVA admixed with CpG could facilitate moderate levels of OT-I CD8+ T-cell proliferation, DCs treated with OVA-loaded and OVA/CpG-co-loaded B16-WT sRCCs facilitated significantly greater levels of OT-I CD8+ T-cell proliferation relative to the respective soluble OVA conditions (Figure 6C). Taken together, these data demonstrate that antigens associated with adjuvant-loaded sRCCs are efficiently presented by DCs in vitro in a functional manner, and that the presentation of these sRCC-associated antigens is significantly more efficient than antigens delivered in a soluble format.

2.4. Immunologic Functionality of Adjuvant-Loaded RCCs In Vivo

The in vivo functionality of the RCCs was evaluated by performing vaccinations of naive mice and measuring antigen-specific cellular and humoral immune responses. First, vaccinations were performed either with B16-WT-derived lysate admixed with OVA protein and MPLA, or with B16-WT sRCCs co-loaded with the same amount of OVA and MPLA. Analysis of T cells in the peripheral blood 8 days post-vaccination showed significantly greater frequencies of functional IFNγ-producing CD8+ T cells in samples from mice treated with sRCCs as compared to mock-treated or lysate-treated mice, following ex vivo stimulation of peripheral blood mononuclear cells (PBMCs) with a relevant MHC I-restricted OVA peptide (Figure 7A). Consistent with this, sRCC vaccination also facilitated the induction of OVA-specific IgG1 and IgG2a antibody titers comparable to or greater than that of lysate (Figure 7B).

Lastly, to evaluate the immune response to endogenous parent cell antigens elicited by sRCCs, naive mice were vaccinated with either B16-mOVA-derived lysate admixed with CpG and MPLA, or with B16-mOVA sRCCs co-loaded with the same amount of CpG and MPLA. Because immune responses can be induced against a diversity of different parent cell antigens, the cellular immune response was evaluated by co-culturing T cells isolated from spleens of treated mice with irradiated B16-mOVA parent cells, and T-cell stimulation evaluated via IL-2 production was measured using Enzyme-Linked ImmunoSpot (ELISPOT). sRCC vaccination facilitated the induction of functional B16-mOVA-specific T cells at frequencies comparable to or greater than that of
lysat (Figure 7C). Taken together, these data demonstrate that RCCs can facilitate the induction of antigen-specific cellular and humoral responses in vivo.

3. Discussion

In this work, we show that whole cancer cells can be processed to produce antigen-rich particles termed RCCs. Although these structures can be generated through various methods, a derivation method based on sonication led to the retention of greater than 20% of the total protein content. Proteins from different intracellular compartments were retained in a distribution similar to that of the parent cells, providing a broad antigen pool for anti-cancer immunization. In contrast, RCCs generated via extrusion using the method employed in this work retained significantly less total protein, with a significant loss of non-membrane-associated protein. The difference in protein retention between eRCCs and sRCCs likely stems from the different mechanisms of cell
disruption that take place during extrusion versus sonication. Cell disruption during extrusion is expected to take place via mild mechanical shear, likely resulting in a brief disruption of the membrane-enclosed compartments as the cells are forced through the pores. In this work, cells were extruded through membranes with a minimal pore size of 1 µm, which is small enough to disrupt membrane-bound organelles, potentially resulting in the release of their contents. Indeed, while the majority of the lipid content was recovered following density centrifugation, the significant loss of cytosolic and nuclear proteins, but not membrane-associated (including nuclear envelope-associated) proteins, suggests that this is the case. An additional factor contributing to protein loss could be fouling of the filter membrane during extrusion. In contrast, cell disruption during sonication takes place primarily via cavitation, involving the continuous mass nucleation, expansion, and implosion of microbubbles throughout the medium, which causes complete disintegration of membranes and the release of soluble protein content. A possible mechanism for the observed retention of soluble protein by the sonicated RCCs could involve the recapture of a fraction of the freed protein during the thermodynamically favored reformation of membrane-enclosed structures following membrane disintegration.

This work also demonstrated that sRCCs can be loaded with both hydrophilic (CpG) and lipophilic (MPLA) adjuvants, illustrating the versatility of this approach. This allows the flexibility to load adjuvants that differentially regulate the generated immune response, as well as diverse combinations of different adjuvants that activate nonredundant signaling pathways. The latter is particularly interesting as it could allow, for example, the generation of RCCs loaded with multiple TLR or other PRR agonists in combinations that mimic natural pathogens. Indeed, it has been shown that the delivery of multiple PRR agonists enhances the resultant cytokine response by immune cells, and that certain combinations show a greater synergy than others. In the vaccination studies evaluating the immune response to endogenous parent cell antigens, combinatorial delivery of CpG and MPLA was employed, allowing for activation of both the MyD88- and Toll-interleukin 1 receptor (TIR)-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways. Further studies exploring the synergy of various adjuvant combinations could allow for further optimization of this system.

It was observed that adjuvant was taken up more efficiently by DCs when it was associated with the sRCCs than

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Figure 7. Cellular and humoral response to sRCC vaccinations in naive mice. A,B) Cellular and humoral response to vaccination with sRCCs or cell lysate derived from B16-WT cells, and loaded or admixed, respectively, with exogenous OVA protein. A) Representative flow cytometry plots with mean frequency of IFN-γ-positive cells among live CD8+ cells in peripheral blood (top), and quantification of data (bottom). Cells were stimulated in vitro with peptide. B) Anti-OVA IgG1 and IgG2a serum titers, on day 8 post-vaccination. Naive mice were vaccinated subcutaneously with either 370 µg B16-WT sRCCs loaded with 70 µg OVA and 90 ng MPLA, or cell lysate derived from the same number of B16-WT cells (5e6) and admixed with the same amount of OVA and MPLA. C) Cellular response to vaccination with sRCCs or cell lysate derived from B16-mOVA cells. ELISPOT analysis of IL-2-producing T cells from spleens of mice following overnight co-culture with parent B16-mOVA cells on day 4 post-vaccination. Naive mice were vaccinated subcutaneously on days 0 and 2 (prime), and days 16 and 18 (boost), with either 370 µg B16-mOVA sRCCs loaded with 18 µg CpG and 180 ng MPLA, or cell lysate derived from the same number of B16-mOVA cells (5e6) and admixed with the same amount of CpG and MPLA. Quantification of spots (left) and representative images of the ELISPOT membranes (right). Values represent the mean ± SD, *p < 0.05, **p < 0.001. The data in (C) was analyzed using one-way ANOVA, followed by Tukey post-testing.
when it was administered in free form. Treatment of DCs with adjuvant-loaded sRCCs enhanced the expression of surface activation markers and the production of the proinflammatory cytokine IL-12 compared to soluble adjuvant, and enhanced the efficiency of antigen presentation compared to soluble antigen sources such as lysate. The enhancement in payload uptake is likely due to the concentration of the adjuvant and antigen in a colloidal vehicle, which is consistent with previous findings. Enhancement of DC activation by CpG, which signals via the endosomal TLR9, is likely related to the enhanced uptake, and is also consistent with previous findings that association of CpG with a colloidal vehicle enhances its adjuvant activity. Enhancement of DC activation by MPLA, which signals via the cell surface TLR4, is likely related to an enhancement of MPLA avidity on the surface of the sRCCs, which can facilitate TLR4 clustering and subsequent activation. Notably, it was observed that non-adjuvanted sRCCs did not activate DCs compared to mock treatment. This demonstrates that non-adjuvanted sRCCs are not inherently immunostimulatory and should not promote adverse inflammatory reactions, for example, due to the retention of endogenous danger associated molecular patterns (DAMPs). Rather, loading the sRCCs with exogenous adjuvants is necessary to impart them with immunostimulatory capacity, and the ability to load these structures with adjuvant using this derivation method is critical to the utility of the generated structures for vaccination.

Collectively, the observations presented herein suggest that adjuvant-loaded sRCCs potentially represent an efficient and versatile cancer vaccine platform. Indeed, previous work employing material isolated from deconstructed cancer cells supports the idea that such material, when appropriately adjuvanted, can be employed as a cancer antigen source for vaccination both in vitro and in vivo. The generation of adjuvant-loaded sRCCs is a method for processing cancer cells in which the antigen diversity of the parent cells is maintained, but the antigen content is reformulated into cell material-derived particles that facilitate efficient antigen presentation. This is in contrast to standard bottom-up design approaches for targeted vaccines, which do not recapitulate this degree of antigen diversity. Notably, sRCC derivation does not involve the input of any exogenous material, and it is therefore expected that the RCCs can be fully degraded by APCs without any harmful degradation products. Further, as simply a method for processing cancer cells, sRCC derivation is analogous to the preparation of tumor lysate, which is also a form of processed cancer cell material, and is the current standard for primary cancer antigen mixtures. Importantly, lysate is commonly employed in diverse cancer vaccine systems in both the preclinical and clinical settings, demonstrating that such cancer cell-derived material is generally considered a safe antigen source for vaccines. Tumor lysate as an antigen source is most commonly used in DC vaccines, vaccines that activate and load DCs with cancer antigen ex vivo for subsequent reinfusion. The in vitro observations reported in this study are compelling evidence that adjuvant-loaded RCCs may represent a superior alternative to tumor lysate in such vaccine approaches by facilitating enhanced uptake and cross-presentation of cancer antigens.

Notably, the in vivo data also suggests that RCCs may be more effective than lysate in this context, although the relatively low frequency of antigen-specific T cells generated against endogenous cell antigens indicates that the RCCs will likely need to be optimized for direct in vivo applications. One suboptimal property of the RCCs for in vivo applications is their broad size distribution, which extends far beyond a feasible size range for passive drainage to the lymph nodes via the lymphatics. In order to optimize the RCCs for stand-alone vaccination, it may be necessary to improve the size distribution of the particles via further processing, for example, through post-sonication extrusion. Alternatively, improving the active trafficking of the RCCs to the lymph node by APCs could be achieved via surface modification with cues that enhance APC uptake, or by incorporating the RCCs into material scaffolds that bring large numbers of relevant APCs to the vaccination site.

4. Conclusions

Whole cancer cells were processed to produce subcellular vesicular particles within the nano- to microscale size range that retain a broad distribution of proteins representative of the parent cells from which they were derived. When these structures, termed RCCs, were loaded with adjuvants, they facilitated enhanced DC activation and antigen presentation when compared to soluble adjuvants and antigens, respectively. Vaccination of mice with these structures also facilitated the induction of antigen-specific cellular and humoral immune responses. Based on these observations, we propose that these RCCs represent an attractive platform for cancer vaccination, in particular, as an alternative to tumor lysate in vaccine approaches that currently use lysate as an antigen source. RCCs could also have utility in various materials-based approaches that function to bring large numbers of APCs to the vaccination site, and may be potentially useful as a stand-alone particulate cancer vaccine system with optimization.

5. Experimental Section

*Animals:* All work with C57BL/6 and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice (The Jackson Laboratory) was performed in compliance with the National Institutes of Health and institutional guidelines.

*Cells and Reagents:* The B16-F10 murine melanoma cell line (ATCC) was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HI-FBS) and 1% penicillin-streptomycin. The MF2.2D murine T cell hybridoma cell line, kindly provided by Dr. Kenneth Rock, was cultured in RPMI 1640 supplemented with 10% HI-FBS, 2 mM L-glutamine, 55 μM beta-mercaptoethanol, 1× non-essential amino acids, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 1% penicillin-streptomycin. The B3Z murine T cell hybridoma cell line, kindly provided by Dr. Nilabh Shastri, was cultured in RPMI 1640 supplemented with 10% HI-FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM beta-mercaptoethanol, and 1% penicillin-streptomycin.
Primary CD8+ T cells were isolated from the spleens of OT-I mice and enriched using an anti-CD8 MACS isolation kit (Miltenyi Biotec). T cells were used immediately for co-culture experiments and were cultured in RPMI 1640 supplemented with 10% Hi-FBS, 1% penicillin-streptomycin, 1 mM sodium pyruvate, 5 mM HEPES, and 50 μM beta-mercaptoethanol.

Cpg and Cpg-FAM (fluorescent Cpg) were synthesized by Integrated DNA Technologies based on the described B class 1826 sequence (ttcatgacgttcctgacgtt). Monophosphoryl lipid A (MPLA) derived from Salmonella minnesota R595 was purchased from Invivogen. Unless otherwise stated, antibodies were purchased from eBioscience.

**Derivation of RCCs:** B16-F10 or B16-mOVA cells were grown in PBS or H2O, respectively. For stability studies, in which DLS measurements were taken.

**Characterization of RCCs:** The RCC hydrodynamic size and zeta potential were evaluated via dynamic light scattering (DLS) using a Malvern Nano ZS. Size and zeta potential measurements were recorded on RCCs suspended at approximately 0.005 mg mL−1 in PBS or H2O, respectively. For stability studies, in which DLS measurements were taken over time, sRCCs were suspended at approximately 0.05 mg mL−1 in either PBS, or DMEM supplemented with 10% Hi-FBS, and stored at 4 °C, or at 37 °C, respectively. At the indicated timepoints, samples were bath-sonicated for 5 minutes, and size measurements were taken.

For quantification of payload loading, payloads were loaded into sRCCs and the purified sRCCs were subsequently lysed in RIPA buffer. Cpg loading was quantified through a combination of fluorescent measurements of a fluorophore-tagged Cpg conjugate using a standard plate reader, and quantification with HPLC. HPLC analysis was conducted on an Agilent 1100 HPLC equipped with an Xterra MS C18 5 micron, 4.6 mm × 250 mm Column (Waters Cat# 186000494). Samples were analyzed using 0.1 m triethylammonium acetate, pH 7 (Mobile Phase A) and 90% acetonitrile in 0.1 m triethylammonium acetate, pH 7 (Mobile Phase B) with the following gradient: 0 min-2%B, 4 min-2%B, 45 min-55%B, 50 min-90%B, 51 min-2%, 60 min-2%. The flow rate was 0.5 mL min−1, the column temperature was 30 °C, the samples were injected without dilution at 50 μL, and detection was conducted at 260 and 488 nm. MPLA loading was quantified through a combination of fluorescent measurements of a fluorescent MPLA conjugate using a standard plate reader, and measurements of untagged MPLA using an Endosafe-MCS system (Charles River). Ovalbumin loading was quantified through fluorescent measurements of a fluorescent ovalbumin conjugate (Invitrogen) using a standard plate reader. The data shown are means of loading studies conducted with fluorescent conjugates. Alternative approaches were performed to ensure consistency of the loading measurements.

**RCC Uptake Studies:** Uptake of RCC-loaded Cpg was evaluated using a 5’ fluorophore conjugated Cpg (Integrated DNA Technologies). Primary DCs were treated with either free or RCC-associated fluorescent Cpg for various lengths of time and then analyzed via either flow cytometry or confocal microscopy for uptake. For flow cytometry analysis of uptake, treated DCs were removed by scraping and stained for viability (Biolegend) and CD11c. Cells were analyzed on a BD LSRFortessa and the percentage of Cpg+ cells among the live CD11c+ cells was quantified. To evaluate uptake via confocal microscopy, cells were analyzed either live or fixed. For live cell imaging, DCs were seeded on glass-bottom tissue culture plates (MatTek). Adjuvant-loaded cells were treated with Hoescht 33342 (Life Technologies) for 15 minutes followed by imaging on a Leica SP5 X MP Inverted Confocal Microscope equipped with a Ludin incubation chamber. For fixed cell imaging, the DCs were seeded on glass coverslips. At the time of analysis, adjuvant-loaded DCs were fixed in 4% paraformaldehyde/PBS for 10 minutes at room temperature followed by permeabilization in 0.1% Triton X/PBS for 15 minutes. Cells were then stained with Alexa Fluor 594-phalloidin (Life Technologies) and Hoescht 33342 (Life Technologies) and mounted on glass slides using ProLong Gold antifade reagent (Life Technologies). The cells were subsequently imaged on a Zeiss LSM 710 confocal microscope.

**DC Activation and Antigen Presentation Studies:** For DC activation studies, adjuvant-loaded sRCCs were prepared at a total protein/adjuvant mass ratio of 1:0.05 for Cpg, and 1.0:002 for MPLA. To evaluate the stimulatory functionality of sRCC-associated adjuvants, primary DCs were plated in non-tissue culture-treated plates, and Cpg (0.2 μM) or MPLA (0.02 μM) was added in free or sRCC-associated form. The Cpg stock used for these studies was tested for endotoxin content and found to have <0.05 EU mg−1 endotoxin contamination. After a 12–15 hour stimulation, media samples were collected for IL-12 quantification via ELISA (Peprotech), and cells were removed by scraping and then stained for viability, CD11c, MHC II, and the costimulatory molecules CD40 and CD86. DC activation was evaluated via flow cytometry based on the proportion of live CD11c+ cells that were positive for the activation markers.

Adjuvant-loaded RCCs were prepared at a total protein/Cpg mass ratios of 1:0.05 for hybridoma T cell and OT-I CD8+ T cell
co-culture studies. To evaluate the capacity of the RCC-delivered antigen to be presented in MHC I and II, primary DCs were plated in non-tissue culture treated plates and treated with either free or RCC-associated antigens for 3 hours. The loaded DCs were subsequently co-cultured with either MF2.2D cells to investigate MHC II presentation, or primary CD8+ OT-I T cells to investigate MHC I cross-presentation, in a total culture volume of 0.2 mL. For co-culture experiments involving the use of the MF2.2D hybridoma line, the co-culture was carried out for 12–15 hours after which the media was collected for IL-2 quantification via ELISA (Peprotech). For CD8+ OT-I T cell co-culture, primary CD8+ T cells were isolated from OT-I mice, as described above, and stained with carboxyfluorescein succinimidyl ester (CFSE) according to the manufacturer's instructions. CFSE-stained T cells were then co-cultured with treated DCs for 3 days. After the co-culture period, the T cells were collected and stained for viability and CD8α and analyzed via flow cytometry. CFSE dilution of live CD8+ cells was evaluated.

**Vaccination Studies:** 6–10 week old C57BL/6j mice were used for all vaccination studies. Mice were vaccinated subcutaneously in the flank, typically with 370 μg of RCCs per dose, or lysate derived from the same starting number of parent cells.

**Ex Vivo Peptide Stimulation and Intracellular Cytokine Staining:** For detection of functional peripheral blood OVA-specific CD8+ T cells, peripheral blood was collected at indicated time points, and erythrocytes were lysed with ammonium-chloride-potassium (ACK) buffer (Lonza). Peripheral blood mononuclear cells (PBMCs) were stimulated with 1 μM SIINFEKL peptide (ACK) buffer (Lonza). Peripheral blood mononuclear cells were stained with live/dead dye (eBioscience) and for surface CD8, fixed/permeabilized, and stained for IFNγ (gating scheme shown in Figure S9, Supporting Information).

**Detection of Serum Anti-OVA Antibodies:** Serum was collected by centrifuging peripheral blood at 550 rcf for 5 minutes. Anti-OVA antibodies were detected by incubating diluted serum with OVA-coated plates overnight, and subsequently detecting relevant IgG subclasses using appropriate anti-mouse secondary antibodies. IL-2 ELISPOT: Spleens were processed by mashing through a 70 μm nylon cell strainer and red blood cell lysing with ACK buffer. T cells were subsequently purified using a pan T-cell negative selection MACS kit (Miltenyi Biotec). The T cells were seeded at 500 000 cells per well in 96-well plates coated with anti-IL2 antibody (BD) and co-cultured for 18 hours with 50 000 irradiated (10 000 rad) B16-mOVA cells that had been treated overnight with 10 ng mL−1 IFNγ to increase MHC I presentation. Captured IL-2 was subsequently detected using an HRP-conjugated IL-2 antibody (BD). Automated spot quantification was performed using a CTL ImmunoSpot S4 analyzer.

**Statistical Analysis:** The statistical analysis was performed using GraphPad Prism. Unless otherwise stated, data were compared using the unpaired two-tailed t test and p-values less than 0.05 were considered to be statistically significant. Where applicable, data are reported as the mean ± SD.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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