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Brief Reviews

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Dendritic Cell–Derived Exosomes as Immunotherapies in the Fight against Cancer

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Exosomes are nanometric membrane vesicles of late endosomal origin released by most, if not all, cell types as a means of sophisticated intercellular communication. A multitude of studies showed how exosomes can mediate and regulate immune responses against tumors. Dendritic cell–derived exosomes (Dex) have received much attention as immunotherapeutic anticancer agents since the discovery that they harbor functional MHC–peptide complexes, in addition to various other immune-stimulating components, that together facilitate immune cell–dependent tumor rejection. The therapeutic potential of Dex has been substantiated with their development and clinical testing in the treatment of cancer. This review focuses on mechanisms by which Dex interact with and influence immune cells and describes how they can be engineered to promote their immunogenic capacity as novel and dynamic anticancer agents. *The Journal of Immunology*, **2014, 193: 1006–1011.**

Cellular secretion of membrane vesicles into the extracellular environment allows modulation of the physiology of recipient cells at a level beyond that of more classical (e.g., cytokine, growth factor) molecular-signaling pathways. This intercellular communication can occur locally or over long distances and affect cells of different lineages. Membrane vesicle signaling involving immune cells can create complex cellular modifications that may play a substantial role in how immune responses are manifested, thus potentially dictating the outcome of infectious diseases and cancer. Following much dedicated research, several novel approaches have been brought to light in which secreted membrane vesicles, namely exosomes, could be targeted or exploited to manipulate the course of various diseases, with cancer being a prominent example and the focus of this review.

Secreted membrane vesicles are composed of a lipid bilayer possessing transmembrane proteins, inside of which is enclosed various cytosolic components and molecules from the donor cell. This structure and composition enable intercellular vesicle signaling to physically change properties of an acquiring cell via transfer of receptors and components of signaling pathways, enzymes, and molecules that can regulate gene expression (e.g., mRNA and microRNA [miRNA] molecules). Several subtypes of vesicles have been documented, each with their own biochemical properties, origination from different intracellular locations, and with certain vesicles being secreted by particular cell types or at particular times in the life of a cell (1). Notable examples include apoptotic bodies of cells undergoing apoptosis, microvesicles that directly bud from the plasma membrane and are usually of a size > 0.2 μm, and exosomes (30–100 nm) secreted following their creation from endosomal membranes within cells. We have limited knowledge as to whether different types of microvesicle have distinct roles in the context of immunity. This is mainly due to ongoing technical difficulties in the discrimination between exosomes and other microvesicles (because differentiation by size, buoyancy density, and protein composition is often insufficient), which may only be solved when we have developed sufficient tools to target the specific molecular machineries involved in formation and cargo sequestration of a given membrane vesicle (2).

The best studied and characterized of these microvesicles in the cancer immunotherapy field are exosomes. Their formation results from the inward budding of endosomal membranes, with the resulting endosome and its content of intraluminal vesicles subsequently referred to as a multivesicular body (MVB) (3). If not targeted for lysosomal degradation, MVBs can fuse with the cellular plasma membrane, allowing release of the intraluminal vesicles as exosomes (3, 4) (Fig. 1). A wide range of immune and nonimmune cell types has been observed to secrete exosomes, including macrophages, dendritic cells (DCs), B cells, CTLs, platelets, mastocytes, fibroblasts, epithelial cells, and tumor cells (1, 5).

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Abbreviations used in this article: CTX, cyclophosphamide; DC, dendritic cell; Dex, DC-derived exosome; iNKT, invariant NKT; mDex, exosome derived from LPS- or IFN-γ–matured DC; miRNA, microRNA; MVB, multivesicular body; TDE, tumor-derived exosome; Treg, regulatory T cell.

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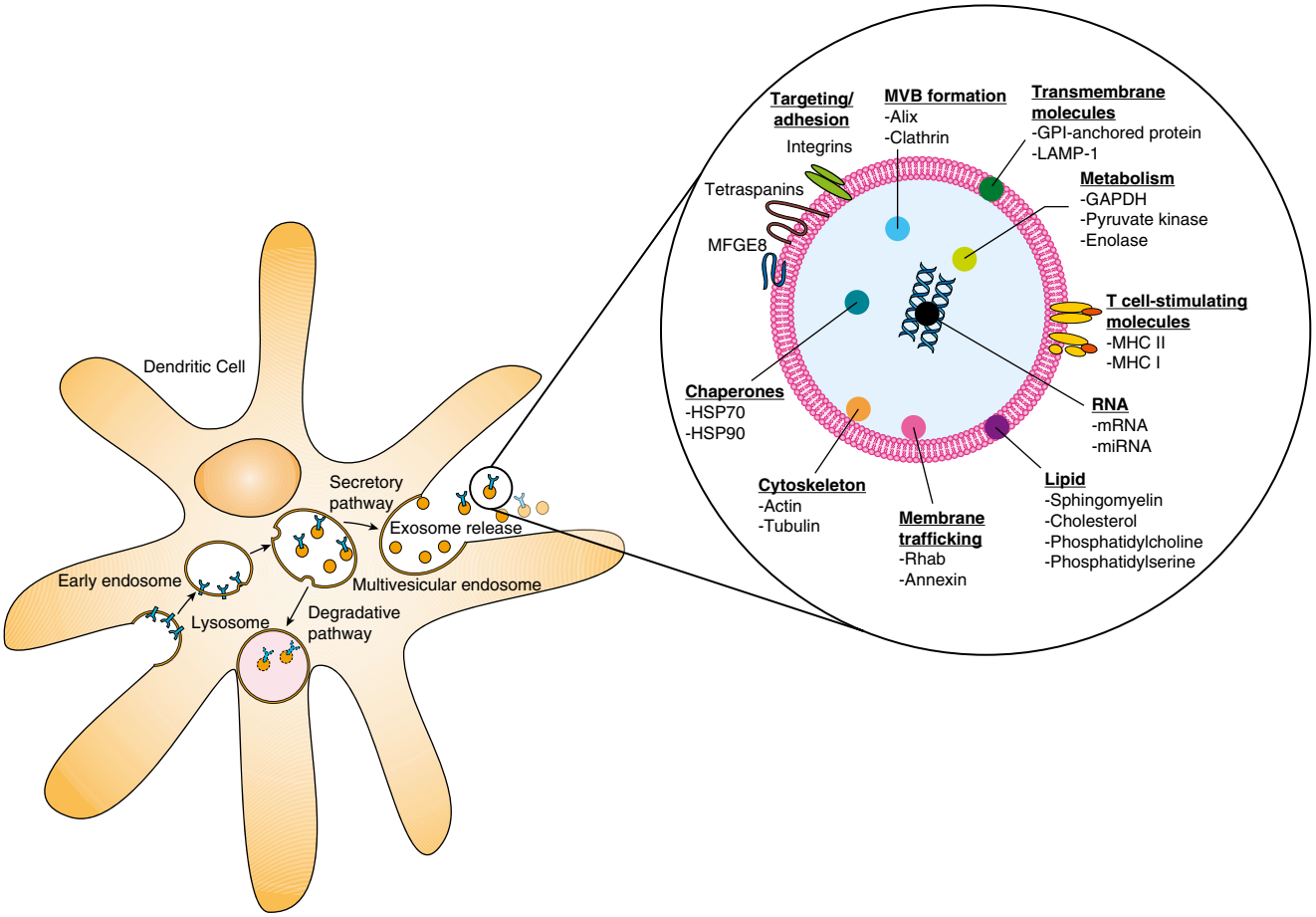

FIGURE 1. The biogenesis and composition of exosomes. Exosomes are generated from the inward budding of endosomal membranes to form MVBs. These are released by fusion of the MVB peripheral membrane with the cell plasma membrane. Exosome composition depends on the cell of origin. Exosomes are limited by a bilipid layer that is enriched with molecules derived from the MVB, such as tetraspanins and MHC. Exosomes contain various cytosolic proteins involved in MVB formation (e.g., Alix), membrane trafficking (Rhab, Annexin), or cytoskeleton organization. They also contain enzymes and RNA molecules that can impact exosome-acquiring cells. MFGE8, milk fat globule EGF factor 8 protein.

Exosomes and cancer

Malignant cancer cells actively drive their own development, progression, and metastasis through modulation of their environment, and it is becoming increasingly apparent that this modulation can occur via tumor-derived exosomes (TDEs), in addition to tumor cell–secreted factors. TDEs were shown in several cancer models to actively promote tumorigenesis and metastasis through mechanisms including modulation of bone marrow progenitors (6), modulation of sentinel lymph nodes (7), and transfer of oncogenic receptors, proteins, and RNA (8, 9). Evidence also suggests that TDEs can actively suppress tumor-specific immune responses. Two studies showed that TDEs carrying the model Ag OVA can suppress delayed-type hypersensitivity responses in OVA-immunized animals. This effect was shown following local administration of TDEs that had been recovered from blood plasma of OVA-immunized mice or from mice harboring OVA-expressing tumors (10, 11). In tumor-bearing hosts, circulating $CD11b^{+}$ exosomes suppressed these tumor Ag–specific responses, but only when the exosomes expressed MHC class II molecules (MHC class II molecules were speculated to stimulate regulatory T cell [Treg] activity) (11).

Immunosurveillance of tumors may similarly be inhibited by TDEs. Pretreatment of mice with TDEs produced by murine mammary tumor cells accelerated the growth of implanted tumor cells, which correlated with a decrease in the number and cytotoxic activity of NK cells ex vivo and in vitro (12). TDE uptake by immature DCs and myeloid precursors was shown, in several models, to block differentiation into mature DCs (13), and it can even promote the induction of myeloid-derived suppressor cells (14). The exact mechanisms determining how these endogenous tumor–derived vesicles suppress antitumor immune responses remain to be fully characterized and may yield important novel therapeutic targets (15). In addition, with the substantial evidence suggesting that TDEs facilitate cancer progression, the monitoring of TDEs may prove to be a useful prognostic and diagnostic biomarker in cancer patients (and is under investigation) (9, 16). In contrast and worthy of mention, TDEs also were reported to contain tumor-rejection Ags that can generate MHC class I–restricted T cell clones in vitro (5, 17). Furthermore, TDEs can drive T cell–dependent cross-protection against syngeneic and allogeneic tumors in vivo (5). Therefore, although driven by the biomarker potential for TDEs, some promising avenues exist for the use of TDEs in future cancer immunotherapy strategies, potentially through harnessing them as a source of cancer cell Ags for antitumor immune responses (18).

A more feasible exosome-based concept, which has surpassed TDE-targeted strategies in the fight against cancer, is the use of

exosomes derived from Ag-prestimulated DCs. Being the sentinel APCs of the immune system, DCs positioned in the proximity of dying cancer cells are pivotal in driving T cell–mediated antitumor responses toward tumor-associated Ags that are deemed as "nonself." Nevertheless, immunotherapy based on DCs per se remains difficult to practice in clinical settings. This is largely due to the significant challenges of implementing such therapies in large numbers of patients, defining quality control parameters, and storing DCs over long periods of time. The use of DC-derived exosomes (Dex) can overcome these technical limitations while still sharing the ability to present Ags directly and indirectly to T cells, as will be discussed.

The composition of Dex determines their therapeutic potential in cancer

Initial studies into the proteome of Dex revealed a unique molecular composition that could allow and support their in vivo functions and endow them with strong immunostimulatory properties (Fig. 1) (19, 20). To favor the targeting, docking, and fusion with acceptor cells, Dex possess a variety of membrane proteins, including the integrin α and β-chains (αMβ2), the Ig family member ICAM-1, and milk fat globule EGF factor 8 (also known as lactadherin) (1, 19, 20). Milk fat globule EGF factor 8 is an abundant protein of Dex membranes that binds externalized phosphatidylserine on the Dex outer membrane and facilitates exosome uptake by forming a bridge between Dex and αvβ3 or αvβ5 integrins present on recipient cells (20). Membrane microdomain organizing proteins, particularly the tetraspanin family of proteins (e.g., CD63, CD81, CD9), are also well defined within the Dex surface membrane, with some of these postulated similarly to participate in exosome–acceptor cell interactions (19, 20).

One of the more striking attributes of Dex surface membranes that relates to their immunostimulatory potential is the possession of molecules involved in Ag processing and presentation. B cell–derived exosomes were the first ones identified to harbor Ag-presentation machinery; peptide–MHC class II complexes present on the exosome surface could induce Ag-specific MHC class II–restricted T cell responses (21). Subsequently, this phenomenon was discovered to be shared by DCs (20, 22). Furthermore, Dex taken from tumor peptide–stimulated DCs could be used to vaccinate mice, resulting in priming of tumor-specific CTL responses that could control or, in some cases, eradicate established murine tumors (22). This fundamental discovery sparked great interest in exploring the application of Dex as clinical immunotherapy by our group and other investigators. In addition to MHC class II, Dex are known to express MHC class I molecules (as are exosomes derived from virtually any cell type) (23, 24) and were described to transfer MHC I–peptide complexes between DCs to allow efficient activation of $CD8^+$ T cells (25). Additionally, Dex were shown to possess the potent costimulatory molecule CD86, which may contribute further toward aiding T cell priming during Ag presentation (22).

With these discoveries of the Ag-presentation capacity of Dex, an obvious question arose as to how Dex present Ags via MHC to T cells—be it directly or indirectly by Dex-acquiring bystander APCs. Several studies verified that Dex can directly stimulate T cells in vitro, although the evidence suggests that Dex are much more efficient in stimulating T cell lines, activated T cells, and memory T cells compared with naive T cells in this setting (4, 21, 26). Additionally, this T cell stimulation occurs with a lower efficiency than that of the parent APC, but it can be improved if Dex are immobilized or their concentration is increased (4, 26). A more efficient means of T cell activation by Dex appears to occur indirectly following Dex interactions with DCs (Fig. 2) (4, 26, 27), and this is likely to be the most fundamental pathway in vivo. Of particular note, it was shown that Dex priming of naive T cells can only occur if APCs are present (27–29). Two key mechanisms have been described for how antigenic peptide–MHC containing Dex could elicit indirect Ag presentation to T cells via an APC. The first of these is a process known as "cross-dressing": Dex merge directly with the surface membrane of an acceptor APC and so transfer their peptide–MHC complexes to the APC membrane to be recognized by T cells without need of further Ag processing. In support of the DC cross-dressing paradigm, one study showed that Dex are able to stimulate naive Ag-specific $CD4^+$ T cells in vivo via DCs deficient in MHC class II, thereby suggesting Dex MHC complex transfer (in addition, the costimulatory molecules CD80 and CD86 were mandatory on the presenting DCs) (27). A second indirect-presentation mechanism is through APC capture and reprocessing of Dex peptide–MHC complexes, resulting in transfer of Ags from Dex MHCs to APC MHCs (29). In addition to these two paradigms, it is feasible that soluble long peptides or whole Ags contained within Dex are degraded and presented by APCs subsequent to Dex uptake.

In addition to the Ag-presenting capability of Dex, other membrane-associated immune-modulating molecules that were described more recently are worthy of mention. It was shown by our group that Dex derived from human immature DCs possess NKG2D ligands upon their surface that can directly engage NKG2D of NK cells, leading to their activation ex vivo (Fig. 2) (30). It was also found that, in vivo, Dex promoted an IL-15Rα–dependent proliferation and NKG2D-mediated activation of NK cells, resulting in enhanced control of metastatic effects by $NK1.1^+$ cells. This is in line with the observation that Dex used as a vaccine in a previous clinical trial could restore the number and NKG2D-dependent function of NK cells in 50% of the patient cohort (30). Another study found that Dex derived from immature human DCs expressed the NKp30 ligand BAT3 on their surface (an intracellular protein usually associated with DNA damage-induced apoptosis), which could similarly activate NK cells (31). Additionally, the surface expression of TNF, FasL, and TRAIL renders Dex capable of NK cell activation and, furthermore, may enable Dex to directly trigger caspase activation and apoptosis in tumor cells (32).

Various cytosolic proteins exist inside of the Dex outer membrane shell of Ag-presenting apparatus and specialized APC-binding molecules. Many of these are related to Dex biogenesis from endocytic compartments of the parent cell (e.g., annexins, RAB proteins, TSG101) or are participants in signal-transduction pathways (e.g., G proteins and kinases). Initial studies of the proteome of Dex also discovered a significant cytosolic fraction of the heat shock cognate protein hsc73 (20). This hsp70 family member, as well as others (including members of the hsp90 family that are also present

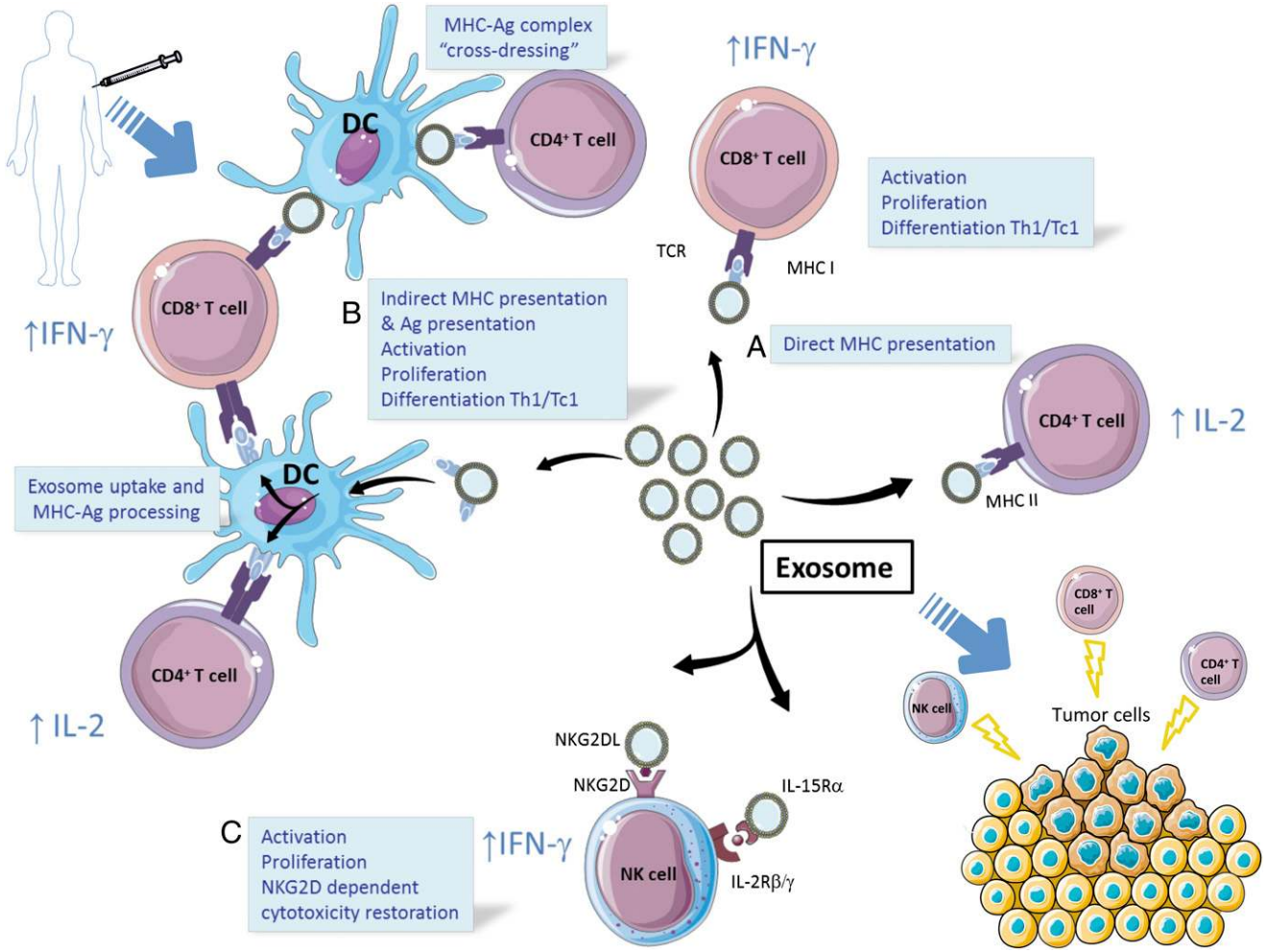

FIGURE 2. Dex interactions with immune cells. Following injection in vivo, Dex can directly activate T cells via direct presentation of peptide–MHC complexes (**A**) or indirectly activate T cells (including naive T cells) via either "cross-dressing" of APCs or exosome uptake and subsequent Ag–MHC processing by APCs (**B**). (**C**) Dex can also promote NK cell activation and proliferation through NK-expressed IL-15Rα and NKG2D.

within Dex), may play a part in Dex immunogenicity, given their Ag chaperone and MHC-loading roles and inherent capacity to activate various immune cells (33).

Finally, exosomes were reported both to contain and transfer mRNA and small RNA (including miRNA) molecules between cells (34). It is believed that the transfer of miRNAs (termed "exosome-shuttle miRNAs") via Dex between DCs can act as a means of communication and posttranscriptional modification, because exosome-shuttle miRNAs could repress target mRNAs of Dex-accepting DCs (35). Thus, posttranscriptional modifications brought about by particular RNA profiles of Dex may influence the outcome of APC functions, with obvious relevance to the success or failure of their clinical application.

Dex in clinical trials

With their sophisticated APC-homing and immunostimulatory properties, in addition to advantages over other cell-based therapies, Dex have been developed for use as cell-free cancer vaccines in the clinical setting. Two phase I clinical trials have been completed in cancer patients (36, 37), each using autologous Dex loaded with MAGE tumor Ags. In one of these studies (37), patients with pretreated advanced MAGE-expressing non-small cell lung cancers received four doses of Dex at weekly intervals. Three of nine patients showed increases in systemic immune responses against MAGE by delayed-type hypersensitivity reactivity (who had no reactivity to MAGE prior to immunization), although only minimal increases in Ag-specific T cell activity were detected. At the trial's conclusion, stable disease was observed in some immunized patients (37). The second clinical study in MAGE3$^+$ advanced melanoma patients (who similarly received four Dex vaccinations at weekly intervals) revealed an objective response in one patient who continued to be stable for 24 mo with Dex therapy continuation, one minor response, and two stabilizations of disease (36). Again, MAGE-specific T cell responses could not be detected in peripheral blood of the patients in the trial, although enhanced NK cell effector functions were detected in 8 of 13 patients (36). Importantly these studies confirmed the safety of Dex administration in patients and highlighted the feasibility of large-scale Dex production, paving the way for further clinical investigation.

The somewhat limited efficacy and insufficient Dex-induced T cell responses in patients in these initial clinical trials may be explained by a combination of several factors. First, these phase I trials could only be performed in advanced-stage patient cohorts, in which there is likely to be a significant immunosuppressive environment to overcome. Additionally, these patients had progressive, not stabilized, disease. Lack of

sufficient T cells in the circulation (i.e., T cell migration to tumors) and inadequate Ag presentation/costimulation also may have hindered therapeutic activity (36, 37). Regarding this latter point, the use of Dex derived from immature DCs without adjuvant, as opposed to matured DCs, may be partly responsible (25, 38).

Studies following the first Dex clinical trials revealed ways to improve the interactions of Dex with the host immune system and, thereby, the potency of Dex in cancer immunotherapy. The use of exosomes derived from LPS- or IFN-γ–matured DCs (mDex) has been an important advance in this field, following discoveries that such Dex induce greater T cell stimulation compared with those from immature DCs (24, 39, 40). This is likely due to the observation that mDex possess more surface MHC class II, ICAM1, and costimulatory molecules (39–41). Also, it was recently found that Dex should be engineered toward stimulating B cell responses, in addition to T cell stimulation, for optimal immunogenicity (42, 43). Indeed, indirect loading of Dex with proteins (i.e., through DC pulsing), but not T cell peptide loading of Dex, induces $CD8^+$ T cell responses and protection against tumor growth in vivo; this occurs only in the presence of B cells and $CD4^+$ T cells (42).

The implementation of this new knowledge in human DC cultures contributed to a second generation of Dex immunotherapies (44). A phase II trial in advanced non-small cell lung cancers patients is underway to investigate whether mDex used as maintenance immunotherapy can ameliorate the rate of progression-free survival at 4 mo postchemotherapy (44).

Dex as part of immunotherapy or combinatorial regimens against cancer

Perhaps the best clinical efficacy against cancer using Dex will be seen, as it is for many other cancer therapies, as part of a combinatorial treatment regimen. We showed previously that combining cyclophosphamide (CTX) chemotherapy with Dex vaccines in vivo can significantly boost tumor- or peptide-induced $CD8^+$ T cell recall responses, leading to potent synergistic effects against pre-established tumors (45). However, addition of CTX did not allow efficient T cell priming by Dex in the absence of adjuvants. Because CTX could abolish the suppressive function of Tregs, it is postulated that Dex/CTX therapy retunes the balance from tumor-induced tolerance toward tumor-induced immunogenicity, resulting in the promising tumor control observed (45). The use of CTX to inhibit Treg-facilitated immune suppression during Dex immunotherapy is being evaluated in the ongoing phase II clinical trial (44).

In addition to the aforementioned use of mDex and inclusion of broad B and T cell stimulation, future Dex immunotherapies may benefit from the addition of certain molecular adjuvants for their implementation as cancer vaccines in patients. Highlighting this, the TLR9 and TLR3 agonists, CpG and dsRNA, respectively, induced Dex-mediated $CD8^+$ T cell priming in vivo, as well as efficient tumor rejection in a melanoma model in HLA-A2–transgenic mice (38). Harnessing invariant NKT (iNKT) cell activity using Dex loaded with the iNKT ligand α-galactosylceramide may also potentiate Dex-induced cancer-specific immune responses. Dex loaded with α-galactosylceramide and the model Ag OVA activated iNKT cells in vitro and in vivo, resulting in a potent multifaceted cellular immune response in mice that decreased tumor growth in an OVA-expressing mouse model of melanoma (46).

Finally, Dex derived from genetically manipulated DCs, engineered to promote anticancer immunogenicity, may lead to key advances. For example, studies by Robbins and Morelli (4) showed how the molecular composition and characteristics of Dex may be manipulated to generate tolerogenic Dex (e.g., through viral vectored transfer of genes encoding immunoregulatory cytokines), with obvious potential uses for immunosuppression of autoimmune diseases.

Conclusions

From the knowledge gained thus far, Dex can be engineered to promote their modulation of immune cells, facilitating or restoring anticancer immune responses. It is also clear that the precise engineering leading to the final composition of Dex as a therapy (including adjunctive therapeutic modulators [e.g., TLR agonist adjuvants and/or chemotherapies]) is crucial to their successes or failures in vivo. Consequently, further advances in Dex immunotherapy rely upon future research focusing on less understood components of Dex and how these components interact with acceptor cells. Prominent examples here include how mRNAs, miRNAs, and cytokines carried by Dex (and lipid mediators produced by Dex at sites of inflammation) influence immune cells and tumors (4, 47).

Furthermore, although many intricate studies of in vitro intercellular exosome signaling and therapeutic advances using in vitro–derived exosomes have been undertaken, far less is known about endogenous immune cell and tumor exosome signaling in vivo. Some progress is beginning to be made in an attempt to uncover the effects of exosome secretion by tumor cells. It was found that exosome release can be reduced by Rab27a knockdown by using certain mammary tumor cell lines stably transfected with a Rab27a-specific short hairpin RNA (48). Injection of one of these cell lines into mice revealed reduced tumor progression and abrogated metastases compared with Rab27a-competent tumors. However, this was not observed with a second cell line, highlighting the variability of exosome functions that appear to depend on the type of tumor and its microenvironment. Despite such pioneering studies, key outstanding questions include: Under what conditions and stages of disease or immune responses are particular membrane vesicles generated in vivo? Do they manifest their effects by targeting particular cell types? Is this targeting local to the source of secretion or at a distinct immune/nonimmune tissue? What is the level of competition between membrane vesicles originating from different sources in vivo, and can this be managed therapeutically? and Which membrane vesicle characteristics determine immunoregulatory versus immunostimulatory outcomes? Regarding this final question, as we learn more about the aforementioned immunosuppressive and cancer-facilitating capacity of TDEs in vivo, strategies to inhibit their release from tumors may emerge to become potent cancer-stabilizing therapies. In view of the clinical and preclinical evidence, we envisage that exosomes, Dex in particular, will make key contributions to the future immunotherapeutic armamentarium in the field of cancer vaccination.

Disclosures

The authors have no financial conflicts of interest.

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