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Induction of effective Anti-Tumour Immunity by targeting Dendritic Cells *in vivo*

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In the 1890s, William Coley, an orthopaedic surgeon from New York developed a novel paradigm for the treatment of cancer. He started to treat his patients with bacterial cultures of *Streptococcus pyogenes* based on reports of cancer regression in a patient who had earlier suffered from an acute infection with *S.pyogenes* [1,2]. Over 10% of patients achieved tumour regression following injection with these "Coley's Toxins", as the treatment came to be called [3]. Thus, the field of immunotherapy was born but it took several decades for scientists to discover the immunological basis of tumour regression in patients treated with Coley's toxins. The ability to evade immune attack is now recognized as a functional hallmark of cancer [4].

Dendritic cells (DC) are classical antigen-presenting cells (APC) that play a crucial role in the adaptive and innate immune response [5,6]. DC are observed in various human and murine tumours and are necessary for priming the immune response to cancer [7]. They are capable of capturing tumour-associated antigens (TAA) from dead or living cancer cells and presenting it on MHC-I and MHC-II to T cells in tumour-draining lymph nodes [8,9]. In particular, the capacity of DC to cross-present acquired TAA to $CD8^+$ T cells is required for the activation of $CD8^+$ cytotoxic T lymphocytes (CTL) that are capable of directly killing neoplastic cells [10]. It is well-known that tumour cells can escape immune attack by various mechanisms of immunosuppression. Several TAA are of self-origin and are recognized as such by T cells preventing immune attack [8] [10]. Also, insufficiently matured tumour infiltrating DC can lead to the induction of T cell anergy or regulatory T cells (Treg). Moreover, the tumour microenvironment is immunosuppressive, expressing molecules and cytokines that shut down effector responses, such as PD-L1 [9,11]. Therefore, immune checkpoint blockade therapy, for example, targeting of the inhibitory cell-surface molecules programmed-death ligand 1 (PD-L1) and programmed death-1 (PD-1), is now recognized as highly effective immunotherapy for various forms of cancer [12,13]. However, most patients treated with these drugs do not achieve durable anti-tumour immunity and clinical response [12]. Thus, research on the use of dendritic cell-based vaccines in cancer is of tremendous value in the quest to achieve long-lasting and protective cancer immunity [11]. DC-based immunotherapy offers the potential to efficiently prime tumour specific CTL thereby working in tandem with immune checkpoint blockade or improve outcomes in patients where PD-1/PD-L1 therapy fails to achieve clinical response. It is also pertinent to mention that T cell co-stimulatory molecules may be targeted directly to mediate T cell activation and effector function [14]. In particular, members of the tumour necrosis factor receptor super family (TNFRSF), such as OX40 (CD134) and 4-1BB (CD137) have been widely investigated for their role in tumour immunotherapy and are currently undergoing clinical trials [15]. In a recent study using the B16 mouse melanoma model, a therapeutic $CD8^+$ response was induced by co-stimulation using OX-40 and 4-1BB

antibodies together with transferred tumor-unrelated $CD4^+$ T helper cells [16]. In addition to their respective ligands, OX40 and 4-1BB are reported to be expressed on dendritic cells [17] and targeting 4-1BB with 4-1BBL transfected cells increased maturation and cytokine production (IL-6 and IL-12) in murine splenic DCs [18]. These findings suggest that in future studies it is important to investigate the effects of co-stimulatory therapies on DC activation to gain a comprehensive understanding of their mechanisms of action.

To date, DC-based immunotherapy has been translated to the clinic primarily for melanoma, prostate cancer, glioblastoma multiforme and renal cell cancer [6]. Most of these treatments involve the use of *ex vivo* manipulated DC. Of these, Sipuleucel-T, an autologous DC product primed with a fusion protein of prostatic acid phosphatase (PAP) and GM-CSF, is one of the most clinically beneficial *ex vivo* vaccines to date [19,20]. However, this approach is associated with a high cost and labour-intensive production techniques [21]. For instance, a recent cost analysis in Belgium for acute myeloid leukaemia patients receiving a DC vaccine showed the cost of a GMP vaccine preparation to be € 20,450 per patient [22]. As such, several studies have focused on approaches that target DC *in vivo* [23]. These approaches may be easy to scale up due to their cost-effectiveness and harbour the potential to induce long-term immunity by activating natural DC subsets [21].

In 1993, Dranoff *et al.* demonstrated that DC could be activated *in vivo* against B16 melanoma in mice by injection of irradiated virally- transduced cells that expressed granulocyte-macrophage colony-stimulating factor (GM-CSF) [24]. Subsequently, pre-clinical studies showed that GM-CSF is essential for DC recruitment, maturation and antigen-presentation, while IL-12 produced by mature DC is capable of activating effector lymphocytes [25,26,27]. GVAX® (Cell Genesys, San Francisco, CA) vaccines consist of irradiated autologous or allogeneic, tumour cells that are virally-transduced with adenoviruses or retroviruses to produce GM-CSF [28]. They have now been tested in clinical trials against various types of cancers and have been shown to elicit anti-tumour immune responses [29]. In an early phase I/II clinical study of patients with prostate cancer, allogeneic GM-CSF cells were tolerated safely and showed increased levels of antibodies to tumour-antigens and the infiltration of $CD1a^+$ DC and $CD68^+$ macrophages at injection sites [30]. However, a phase III trial using allogeneic GVAX® was observed not to be superior to current treatments. Nevertheless, studies continue to investigate the use of GM-CSF to prime anti-tumour responses in other cancer types [29,30,31]. A series of seminal papers have also demonstrated the complex tumour microenvironment that must be modulated to achieve long-term anti-tumour immunity. Wada *et al.* showed in an autochthonous prostate cancer model, that cyclophosphamide could promote anti-tumour immunity by transiently depleting Treg in the tumour draining lymph nodes but not those in peripheral circulation [32]. Studies in human cases of pancreatic ductal adenocarcinoma (PDAC) using an allogeneic GVAX® in combination with low-dose cyclophosphamide to deplete regulatory T cells (Treg), induced intra-tumoural tertiary lymphoid aggregates. Post-GVAX T-cell infiltration and aggregate formation resulted in the up regulation of immune-regulatory mechanisms (e.g. PD-1-PD-L1), suggesting that they may be better responders to immune checkpoint and other immune modulatory therapies than vaccine naïve tumours [33]. These observations suggest a crucial role for GM-CSF due to its direct stimulatory effects on DC. The aforementioned studies also provide insight into the design of combinatorial immunotherapies to overcome the various suppressor mechanisms at play in the tumour micro-environment: Given these considerations, a recent phase I trial was conducted using tumour-associated peptides (TUMAP) from renal cell cancer in combination with GM-CSF and preceded by a single-dose of cyclophosphamide [34]. Treatment with the IMA901 vaccine, comprising 9 HLA class-I restricted TUMAP, in combination with GM-CSF and cyclophosphamide resulted in prolonged survival [35]. This was the first study to report a discernable clinical efficacy associated with an anti-tumour peptide vaccine. The clinical response was also shown to be associated with reduced numbers of Treg. A phase III trial is currently underway to further study the therapeutic efficacy of the IMA901 vaccine in patients [34]. The success of this treatment shows the need for eliciting a multifaceted immune response to achieve clinical responses. The anti-tumour immunity induced by GM-CSF is favourable in some forms of cancer and unfavourable in others by inducing a suppressive phenotype [36]. Thus, there is a continued need for agents that can target DC selectively and with high specificity.

Hawiger *et al.* first demonstrated in 2001 that antigen could be directly targeted to DC *in vivo* [37]. The authors developed a fusion protein of hen-egg lysozyme (HEL) and a monoclonal antibody targeted to DEC-205, an endocytic receptor of the C-type lectin receptor (CLR) family. It was found that antigen could be delivered to DC which were then processed and presented efficiently to 3A9 transgenic T cells [37]. However, these T cells could not produce Th1 cytokines and after 7 days, became unresponsive to systemic challenge with the antigen. In comparison, concurrent treatment with anti-CD40 antibodies resulted in long-term T cell activation demonstrating the importance of secondary signals for DC maturation [37]. In mice, targeting HIV gag p24 to other CLRs such as Langerin, and Clec9A induced a $CD8^+$ and Th1 immune response if co-treated with anti-CD40 [38]. Targeting to other receptors such as XCR1, which are expressed on cross-presenting $CD141^+$DC in humans and $CD8^+$DC in mice, is an additional approach to induce not only $CD8^+$ but also Th1 responses [39]. Given the number of potential targets and adjuvants capable of modulating DC function *in vivo*, the use of nanoparticles warrants further consideration. Nanoparticles are nano-sized drug delivery systems that can be manipulated in a number of ways to boost cancer immunotherapy [40,41,42]. First, nanoparticle size and surface composition can be selectively designed to increase delivery to specific tissues and control systemic distribution. Second, the desired therapeutic can be encapsulated, embedded or conjugated to the surface. Thus, they can potentially serve as artificial APC that contain both antigens and co-stimulatory molecules on

their surface [41]. Third, nanoparticles can be designed with immunostimulatory biomaterials thus serving as both drug carriers and adjuvants [40,41]. Finally, nanoparticles are capable of sustained release of encapsulated substances providing a long-term therapeutic option and obviating the need for continuous treatment. Encapsulating cytotoxic anti-cancer drugs can further boost immunotherapy by releasing tumour-associated antigens for uptake and cross-presentation by DC. Hence, nanoparticles are multi-functional platforms that are in theory more cost effective, scalable and versatile than *ex vivo* manipulated DC products. DC-targeted nanoparticle systems have not been widely studied but reports from the literature have shown their efficacy and applicability for immunotherapy. In January 2017, Shi *et al*. demonstrated that chitosan nanoparticles loaded with tumour cell lysates and targeted to DC by coating the surface with mannose resulted in DC activation and induction of anti-tumour CTL responses [43]. Last year, a study reported the efficacy of RNA-nanoparticles in a phase I clinical trial in 3 patients [44]. The authors described the generation of RNA-lipid lipoplexes (RNA-LPX), that protected RNA *in vivo* and targeted it efficiently to various lymphoid DC and macrophage subsets. RNA-LPX encoding for the tumour antigens NY-ESO-1, MAGE-A3, tyrosinase and TPTE were used in 3 patients with advanced melanoma. IFNα and antigen-specific T cell responses were observed in all patients [44]. One patient had undergone resection of metastatic lesions and thus was tumour-free at the time of the submission of the manuscript, while the two other patients demonstrated regression and stable disease respectively [44]. This vaccine could be given systemically and was not associated with any major adverse effects. This was the first study of its kind using a RNA-nanoparticle system which may have broad applications for tumour immunotherapy. First, RNA can be used to encode several tumour antigens and neoantigens. Second, studies in mice with RNA-LPX showed a wide systemic distribution of the nanoparticle to lymphoid DC, potentially increasing the number of responding cells and thus the potency of the treatment [44].

The global burden of cancer continues to rise and immunotherapy heralds the promise of long-term control and eventual cure of the disease. Using DC vaccines to mediate anti-tumour immunity is now deemed a crucial factor for the success of combinatorial immunotherapy [7]. However, the prohibitive costs of GMP-grade manufacturing of human cell products preclude their use in many types of cancers and in many countries of the world. Alternatively, nanoparticles are biocompatible, low-risk and low-cost delivery platforms for immunotherapy. Moving forward, it will be essential to design multifunctional nanoparticles or "nanoparticle cocktails" that are capable of key immunotherapeutic functions. These include effectively targeting DC (e.g. DEC-205), inducing maturation (e.g. anti-CD40), delivering TAA (e.g. RNA or tumour lysates), checkpoint blockade (e.g. anti-PDL1) and finally, inducing tumour cytotoxicity (e.g. doxorubicin). Finally, an additional advantage of nanoparticles is their ability to be loaded with imaging agents [45,46]. This can allow oncologists to measure the infiltration of these nanoparticles in lymphoid tissue and analyze their interactions with immune cells to accurately determine the efficacy of the treatment. Therefore, further research in the context of immunotherapy stands to benefit significantly with these platforms and their inclusion in clinical trials promises to deliver low-cost multiparameter treatments that are essential for precision medicine.

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