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Presentation of tumour antigens by dendritic cells and challenges faced

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The use of dendritic cells (DCs) for the generation of anti-tumour immunity has been the focus of a vast array of scientific and clinical studies. The ability of DCs to present protein tumour antigens (T-Ags) to $CD4^+$ and $CD8^+$ T cells is pivotal to the success of therapeutic cancer vaccines. DC's specialised capacity to cross-present exogenous Ags onto major histocompatibility (MHC) class I molecules for the generation of T-Ag-specific cytotoxic T lymphocytes (CTLs) has made these cells the focal point of vaccine-based immunotherapy of cancer. However, although DC-based strategies can induce T cell responses in cancer patients, recent reviews of clinical studies demonstrate that DC-based approaches have essentially failed to meet their clinical end points. These findings highlight the need to re-evaluate the DC-based vaccine strategies and incorporate recent advancements in DC biology and tumour immunology. The current review considers the issues related to how best to target the Ag-processing pathway of DCs, the role of adjuvants, the appropriate conditioning of the DCs and strategies to overcome tumour-mediated immune escape.

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Introduction

Dendritic cells (DCs) are a family of specialised antigen (Ag) presenting cells (APCs) that form sentinel networks for the detection of tissue damage, pathogen entry and inflammation. However, whilst DCs can detect pathogens such as viruses and bacteria via an array of pattern recognition receptors (surface, endosomal and cytosolic), recognition of malignantly transformed cells is more problematic and likely more dependent on the Ag presentation pathway for immune detection. This system screens all synthesised proteins within a cell and displays signature peptides of each protein (normal, viral or tumour-specific) on the cell surface as major histocompatibility (MHC)/peptide complexes.

DCs acquire Ags by different endocytic routes such as phagocytosis, macropinocytosis, micropinocytosis (fluid phase uptake) and receptor-mediated endocytosis (e.g. antibody-Ag immune complexes via FcRs or C-type lectins, see Figure 1). In this way, DCs ingest exogenous Ags into endosomes and/or phagosomes and can process these for class II MHC presentation to $CD4^+$ T cells or translocate these into the cytosol so as to enter the class I MHC processing pathway for 'cross-presentation' to tumour-specific $CD8^+$ T cells [1]. DCs can naturally take up tumour antigens (T-Ags) *in vivo* either as dead or dying tumour cells, tumour lysates or T-Ag immune complexes [2]. Strategies to harness these capabilities in vaccine-based immunotherapy are examined later.

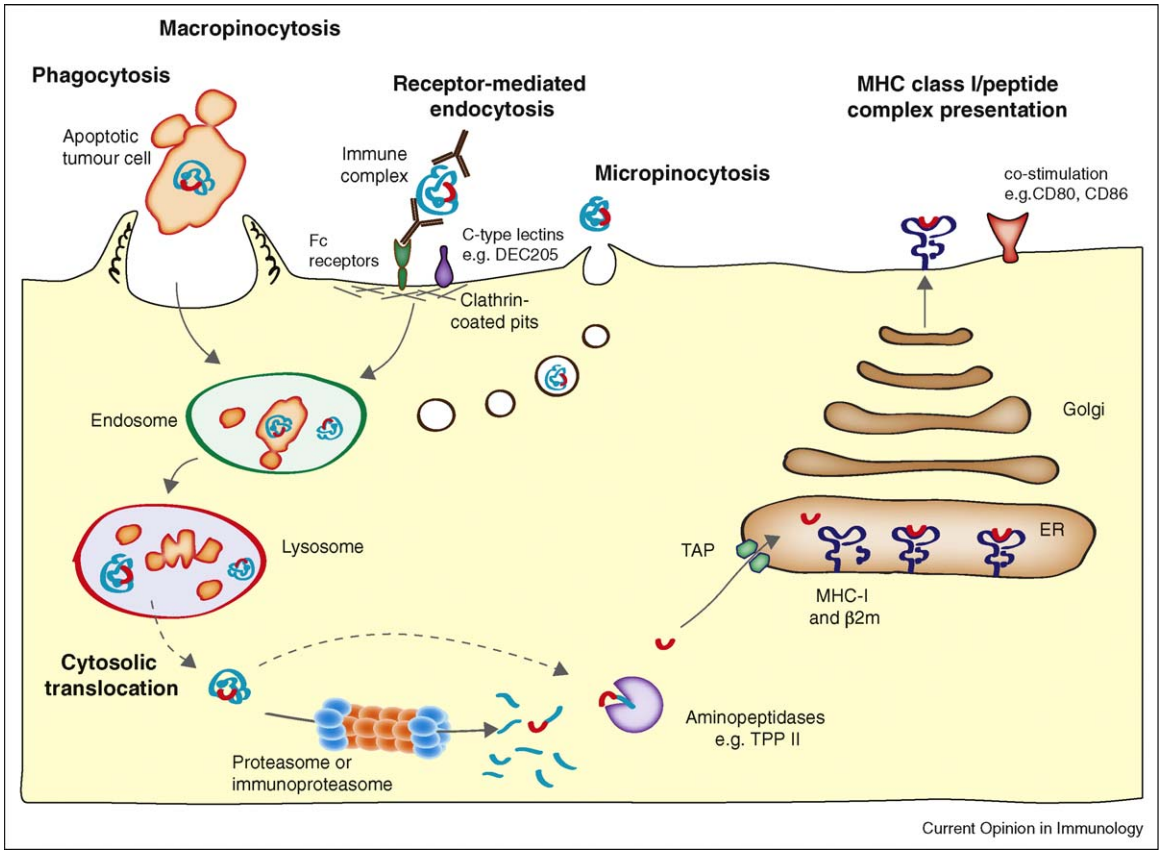
Presentation of tumour Ags by DCs on MHC class I and II

A prerequisite for the lysis of tumours by specific cytotoxic T lymphocytes (CTLs) is the recognition of peptides presented by the tumour cells themselves. However, the initial generation of $CD4^+$ T helper cells and $CD8^+$ CTLs requires Ag-specific activation by professional APCs such as activated DCs that provide co-stimulatory signals (e.g. CD40, CD80 and CD86) and cytokines (e.g. IL-12p70) for expression of full T cell functions.

MHC class II-restricted T-Ag-specific $CD4^+$ T cells are far less described in the literature and analytical tools such as tetramers loaded with peptides for their detection are not commercially available. However, MHC class II expression and presentation of exogenous Ags by immature DCs is constitutive with transient presentation on the cell surface and accumulation in endosomal compartments [3]. During maturation, MHC class II-peptide complexes accumulate on the DCs surface, providing stable display of Ags to activate $CD4^+$ T cells. Importantly, $CD4^+$ T helper cells support the generation of anti-tumour CTL memory responses [4].

Immunotherapeutic approaches with T-Ag loaded DCs predominantly focus on the activation of CTLs that requires Ag to access the MHC class I pathway. *In vivo*, DCs acquire soluble or cell-associated exogenous Ags and

Figure 1



Tumour Ag processing by DCs. *Abbreviations*: β2m, β-2 microglobulin; ER, endoplasmatic reticulum; MHC, major histocompatibilty complex; TAP, transporter associated with Ag-processing; TPP II, tripeptidyl peptidase II.

cross-present these on MHC class I molecules. Antigenic peptides are presented by immature DCs after proteasomal processing and further trimming by aminopeptidases [5]. Activation of DCs by danger signals or cytokines (such as IFN-γ, initially provided by activated NK cells [6]) results in the enhancement of the Ag-processing machinery (APM) by converting proteasomes to immunoproteasomes by introducing different catalytic subunits (LMP2, LMP7 and MECL1) as well as upregulation of the proteasome activator PA28 [7]. The switch from a constitutive proteasome to an immunoproteasome results in accelerated generation of peptides with mainly hydrophobic and basic C-terminal residues [8], which preferentially bind to TAP1/TAP2 heterodimers facilitating translocation into the endoplasmic reticulum (ER) and finally binding to the MHC class I peptide binding cleft [9], thus enabling DCs to activate CTLs more efficiently. Therefore, clinical strategies for DC vaccines should combine Ag delivery with adjuvant-mediated effects to induce DC maturation for efficient priming of CTL responses. Although this paradigm is accepted for most T-Ags, certain T-Ag epitopes such as the HLA-A2-restricted Melan-A/MART-1 epitope (and others) were only found to be cross-presented by the constitutive proteasome and destroyed by the immunoproteasome [10,11].

On the tumour side, defective Ag-processing or presentation is one of several immune escape mechanisms detected in solid tumours and certain haematological malignancies [12,13•,14], resulting in impaired peptide presentation by the tumour and failed recognition by CTLs [15]. The use of histone deacetylase inhibitors has been shown to upregulate components of the APM in tumour cells, thus restoring MHC/peptide expression and facilitating tumour recognition by Ag-specific T cells [16].

Tumour antigens as targets

T-Ags are defined as mutated or abnormally overexpressed proteins in cancer cells. A multitude of T-Ags have been described and new T-Ags continue to be identified. These encompass subclasses such as differentiation T-Ags (e.g. Melan-A/MART1, Tyrosinase, gp100, PAP, PSMA, PSA, CEA), Cancer/Testes T-Ags (CT-Ags, e.g. MAGE family, NY-ESO-1), viral T-Ags (e.g. HCV core, EBVL1 and HPV16 E6E7) and neo-T-Ags to name a

few [17••]. Table 1 lists T-Ags that have shown immunogenicity in cancer patients and are being evaluated in phase II and III clinical studies (http://clinicaltrials.gov).

CT-Ags are the focus of several vaccine-based immunotherapeutic strategies as they show restricted expression in germ cells (e.g. testes and ovaries), are silenced in differentiated somatic tissues and are re-expressed in a wide variety of genetically unstable tumour cells (partially as a result of defective methylation). Unlike differentiation Ags which are expressed in normal adult cells (and thus potentially immune-tolerated), CT-Ags appear to be expressed either before the establishment of the immune response (i.e. embryonic pre-thymic development) or in immune-privileged sites (e.g. testes) and thus are essentially seen as neo-T-Ags to the adult immune system when encountered systemically. Examples of CT-Ags being targeted to DCs or by vaccine approaches include the MAGE family, NY-ESO-1 and LAGE [18].

As mentioned, there is an increasing list of T-Ags reflecting dysregulation of protein synthesis/function in tumour cells. For instance, alterations in phosphorylation of cellular proteins are frequently associated with malignant transformation and recent studies demonstrate that phosphopeptide-specific CTLs are detected in cancer patients [19]. Other new T-Ag targets include serine/threonine kinases (e.g. Aurora-A) [20], protein kinase Cβ [21], viral T-Ags such as HERV type E [22], minor histocompatibility Ags such as HA-1 [23] as well as aberrantly expressed growth factor receptors such as fibroblast growth factor receptor 3 [24]. Finally, inhibitors of cellular function (e.g. against Metnase or Syk) may induce tumour cell apoptosis precipitating the release of T-Ags and resulting in spontaneous anti-tumour immunity [25,26]. In this regard, there is growing evidence that specific classes of conventional therapies (e.g. radiotherapy and certain cytotoxic agents such as Oxaliplatin and Doxorubicin) can facilitate spontaneous T-Ag-specific immunity by inducing immunogenic tumour cell death [27••,28••,29]. This highlights that vaccine-based approaches maybe enhanced when combined with appropriately selected chemotherapy regimens.

Processing of tumour antigens by DCs: roles of protease complexes

Ag cross-presentation by DCs requires that the Ag escapes the endosomal compartment to gain access to cytosolic proteases for further degradation (Figure 1). Cytosolic proteasome complexes play a major role in the initial Ag breakdown for the generation of MHC class I peptides. In addition, a number of other cytosolic peptidases such as puromycin-sensitive aminopeptidase, bleomycin hydrolase, leucine aminopeptidase and thimet oligopeptidase have been implicated to act as trimming enzymes. However, their direct role in the processing and generation of MHC class I peptides remains controversial [30–32]. This is also the case for tripeptidyl peptidase II (TPP II), a large subtilisin type serine protease which exhibits both endopeptidase and exopeptidase activities [33]. Interestingly, TPP II which is expressed by a variety of cells, including human DCs [34•], can generate CTL epitopes that are generally insensitive to proteasome inhibition (e.g. HLA-A3-restricted and HLA-B27-restricted epitopes). Seifert *et al.* first reported that TPP II alone could produce a HLA-A3-restricted HIV nef epitope in DCs [35••], indicating that TPP II may substitute for the proteasome in certain circumstances. Reits *et al.* demonstrated that TPP II mainly trimmed epitope precursor peptides generated by the proteasome [36]; however, this was not observed by others [37]. To add further complexity, we found that targeting full-length NY-ESO-1 protein formulated either as an immune complex or with ISCOMATRIX™ adjuvant to human DCs resulted in cross-presentation of an HLA-A2-restricted epitope via proteasomes or TPP II, respectively [34•,38•]. Furthermore, the protease complexes responsible for generating the NY-ESO-1/HLA-Cw3 and HLA-B7 epitopes also appear to differ, highlighting that the various protease complexes handle epitopes within the same T-Ag differently and this can be modified further by the method of delivery/formulation (NC Robson *et al.*, unpublished data). Whether targeting Ag to distinct cytosolic processing pathways influences the epitope repertoire displayed by DCs and subsequent anti-tumour CTL responses is the focus of further study.

Use of dendritic cells *ex vivo* to deliver tumour antigens in cancer patients

Numerous clinical studies have evaluated DC-based immunotherapy to induce clinically effective immune responses in various cancer types. These have evaluated a range of T-Ags as well as used varying formulations such as defined MHC class I peptide epitopes, full-length recombinant proteins, specific or total tumour cell mRNA, virally delivered DNA, autologous tumour cell lysates or whole tumour cells and even allogeneic tumour cell lines (Table 2 [2,39]). Other variables tested include route of administration (e.g. subcutaneous, intramuscular, intradermal, intravenous and intranodal) and cytokine adjuvants (GM-CSF, IL-2, IFN-γ). The types of DCs used have also varied (e.g. monocyte-derived or blood DCs) as has their maturation status (immature or matured with various cytokines and adjuvants). A recent review of over 38 clinical studies involving 626 patients disappointingly indicates only minor statistical benefits of using peptide-pulsed DCs in cancer patients highlighting the challenges that still face the field [40]. These disappointing clinical outcomes likely reflect firstly, the predominant use of defined peptide vaccines (e.g. HLA-A2-restricted epitopes) that may not be the most immunodominant for any given T-Ag or individual; secondly, the method of DC maturation and thirdly, the poor peptide

Table 1

Current Phase II and III DC-based immunotherapy trials in cancer

Antigen class	Antigen	Clinical trial	Tumour entity	Clinical Investigator/ Commercial Partner	Adjuvant
Cancer/Testis antigens	NY-ESO-1	Phase I	NY-ESO-1$^+$ solid tumours	Celldex, Ludwig Institute	NY-ESO-1-anti-DEC205 mAb
		Phase II	NY-ESO-1$^+$ solid tumours	CSL, Ludwig Institute	ISCOMATRIX^{TM}
		Phase II	NY-ESO-1/LAGE-1$^+$ tumours	GSK, Ludwig Institute	AS15
		Phase II	Ovarian, Peritoneal cancer	Roswell Park Cancer Institute	Fowlpox + vaccinia
		Phase II	Melanoma	H. Lee Moffitt Cancer Center	DCs
		Phase III	Multiple Myeloma	University of Arkansas	GM-CSF
	MAGE A3	Phase III	NSCLC, Multiple Myeloma	GSK University of Arkansas	AS15 (QS21 + MPL + CpG) +GM-CSF
Mutated antigens	p53 mutant	Phase II	Several	NCI	DCs + IL-2 + GM-CSF
	Ras mutant	Phase II	Colorectal cancer, Cervical cancer and others	NCI	DCs + IL-2
	BCR/ABL	Phase II	Chronic Myeloid Leukemia	Memorial Sloan-Kettering Cancer Center	QS21
Differentiation antigens	CEA	Phase II	Colorectal cancer	Lombardi Cancer Research Center	ALVAC + Vaccinia
		Phase II	Colorectal cancer	Duke University	DCs
		Phase II	Breast cancer	NCI	Fowlpox- + Vaccinia-TRICOM + GM-CSF
	gp100	Phase III	Melanoma	Goshen Health System	IL-2
		Phase III	Melanoma	EORTC	None
		Phase III	Melanoma	ECOG	GM-CSF + IFA
	Melan-A/MART-1	Phase III	Melanoma	ECOG	GM-CSF + IFA
	Tyrosinase	Phase III	Melanoma	EORTC	None
	PSA	Phase II	Prostate cancer	University of Iowa	Adenovirus
				ECOG	Fowlpox-, Vaccinia-TRICOM + GM-CSF
		Phase II	Prostate cancer	Duke University	DCs
	PAP	Phase III	Prostate cancer	Dendreon	PAP-GM-CSF + DCs
	PSMA	Phase II/III	Prostate cancer	Northwest Therapeutics	BCG + IFN-γ + DCs
Over-expressed antigens	hTERT	Phase III	Acute Myeloid Leukemia	Geron Corporation	RNA transfected DCs
	MUC1	Phase III	NSCLC, Breast cancer	Merck KGaA/Oncothyreon	Liposome + Vaccinia-IL-2
		Phase III	NSCLC	Transgene	MVA-MUC-1-IL-2
		Phase I	Breast cancer	Prima Biomed	MUC-1-mannan
	HER2/neu	Phase II	Breast cancer, Colorectal cancer	University of Pennsylvania Duke University/GSK	DCs + AS15 adjuvant
	Telomerase	Phase III	Pancreatic cancer	Royal Liverpool University Hospital	None
	WT1	Phase II	Hematological cancers and others	NCI	DCs
	p53	Phase II	NSCLC, Ovarian and others	NCI and others	DCs + Adenovirus
	PR1	Phase III	Acute Myeloid Leukemia	Vaccine Company	IFA + GM-CSF
Viral antigens	EBV (LMP2)	Phase II	Head and Neck cancer	NCI	IFA + DCs
	5T4	Phase II	Renal cell cancer, Colorectal cancer	Oxford BioMedica	Vaccinia
	HPV (E6, E7)	Phase III	Cervical cancer	Merck, GSK	VLPs + AS04
Autologous antigens	Autologous	Phase II	Renal cell cancer	Argos	RNA transfection of DCs
	Autologous	Phase II	Colorectal cancer	Dandrit Biotech	Lysates + DCs
	Autologous	Phase II/III	Glioblastoma Multiforme	Northwest Therapeutics	Lysates + DCs + BCG + IFN-γ

Examples of human tumour antigens in phase II or III clinical evaluation.
Abbreviations: BCG, bacille Calmette-Guérin; BCR/ABL, breakpoint cluster region/Abelson proto-oncogene; CEA: carcinoembryonic antigen; GM-CSF, granulocyte/macrophage colony-stimulating factor; HPV, human papillomavirus; hTERT, human telomerase reverse transcriptase; IFA, incomplete Freud's adjuvant; LMP2, latent membrane protein-2; MAGE, melanoma antigen; MLP, monophosphoryl lipid A; MUC1, Mucin-1; NSCLC, non-small cell lung carcinoma; NY-ESO-1, New York esophageal squamous cell carcinoma antigen; PAP, prostatic acid phosphatase; PR1, Proteinase 3 antigen; PSMA, prostate specific membrane antigen; PSA, prostate specific antigen; WT1, Wilms' tumour antigen 1.

half-life on *ex vivo*-pulsed DCs. In this regard, we previously reported that loading myeloid human DCs (monocyte-derived DCs or CD1c$^+$ blood DCs) with full-length protein Ag (particularly when formulated with ISCOMATRIX^{TM} adjuvant) was superior to exogenous peptide loading and resulted in prolonged and highly efficient MHC class I and II presentation for up to three days [34•,38•]. Interestingly, the most promising clinical data

Table 2

T-Ag formulation strategies under clinical evaluation

Form of T-Ag	Adjuvant/formulation	Advantages	Disadvantages
Whole cells or lysates (autologous, allogeneic)	GM-CSF, IL-2, BCG, IFN-γ, Hsp96	Class I and II epitopes Individualised T-Ags No HLA-restriction	Self-antigens (autoimmunity?) Immunosuppressive molecules Undefined antigens (monitoring issues)
Protein	ISCOMATRIX™, QS21™, AS15™ (MPL™ and CpG™), Immune complexes (mAb), antibody-T-Ag fusion constructs (anti-DEC205)	Class I and II epitopes Long-lived class I presentation No HLA-restriction Immunomodulation and DC maturation/activation	Requires processing by APCs Multi-component strategy (complex) Regulatory hurdles (Safety?)
Peptide	IFA (Montanide), GM-CSF, R837 (Imiquimod)	No processing by DCs required Immunodominant epitopes	Short-lived class I binding HLA-restriction (patient selection) Limited class II epitopes DC *in vitro* culture Issue with DC homing *in vivo*
mRNA	Transfection of DCs	Class I and II epitopes Easy access to class I processing No HLA-restriction Individualised T-Ags	DC *in vitro* culture DC transfection efficiency Issue with DC homing *in vivo* Self-antigens (autoimmunity?) Undefined antigens (monitoring issues)
DNA (adenovirus, vaccinia and fowlpox virus)	Viral PAMPs, co-expression of co-stimulatory molecules (TRICOM) ± DCs	Class I and II epitopes Easy access to class I processing No HLA-restriction High immunogenicity	Anti-vector immunity Need to alternate vaccine type Multi-component strategy (complex) Regulatory hurdles (Safety?)

Cancer vaccine types and formulations in clinical evaluation.
Abbreviations: BCG, bacille Calmette-Guérin; GM-CSF, granulocyte/macrophage colony-stimulating factor; Hsp, heat shock proteins; IFA, incomplete Freud's adjuvant; MLP, monophosphoryl lipid A; mAb, monoclonal antibody; PAMP, pattern-associated molecular pattern.

have been derived from Sipuleucel-T therapy (Provenge), using fresh autologous blood DCs pulsed with full length, prostatic acid phosphatase-GM-CSF fusion protein (PAP-GM-CSF). Two separate Phase III studies demonstrated improved overall survival in patients with metastatic, castration resistant, prostate cancer [41]. Provenge has recently been submitted for a Biologics License Application (BLA) that, if approved, would make Provenge the first in the class of Active Cellular Therapies. The complex Provenge process may hold insights into what is required to generate DC-mediated immunity in cancer patients.

Use of adjuvants and delivery strategies to target DCs *in vivo*

A simpler alternative to *ex vivo* loading of DCs with T-Ags is to target them *in vivo* using adjuvants. Adjuvants are components that, when added to subunit Ag-vaccines, boost their immunogenicity and thus immune efficacy. Adjuvants can elicit their effects via two broad mechanisms: first, Ag delivery or depot and second, immunomodulation. Clinically approved adjuvants, such as aluminium salts (Alum), the MF59 oil-in-water emulsion or AS04 (MPL™ with Alum) utilise aspects of both these categories although they depend more heavily on their delivery/depot capability [42–45]; and although DCs are likely engaged *in vivo*, poor CTL responses are generated. In contrast, Toll-like receptor (TLR) agonists (e.g. TLR4 agonist MPL™, TLR7/8 agonist 3M-012™ and TLR9 agonist CpG™) [46–48] are predominantly immune response modifiers of the vaccine Ag lacking delivery/depot capacity but clearly capable of DC activation and CTL generation *in vivo*. The saponin-based ISCOMATRIX™ adjuvant facilitates efficient Ag delivery for presentation and cross-presentation by DCs [38•] as well as immunomodulation for enhanced and accelerated generation of $CD4^+$ and $CD8^+$ T cell responses of broad specificity as well as robust Ab responses [49]. Other adjuvant approaches in clinical development include the use of heat shock protein (hsp96) fractions from patient's own tumour cells [50] and variations on the mRNA/DNA transfection approach such as vaccinia virus encoding GM-CSF [51], or multiple co-stimulatory molecules (CD80, ICAM-1 and LFA-3) [52]. These provide the necessary 'danger signals' that activate DCs and facilitate CTL responses. However, even these have met with mixed clinical outcomes suggesting more is needed. Combinations of various adjuvant components are also being evaluated. The AS series of saponin-based adjuvants (e.g. AS01, AS02, AS15) are at various late stages of clinical development and are composed of the QS21 saponin combined with various TLR agonists (e.g. MPL™ and/or CpG) [53]. Earlier stage adjuvant strategies take advantage of pathogen-based vectors to provide both Ag and immunomodulation. These include killed but metabolically active recombinant *Listeria monocytogenes*

expressing T-Ags [54•]. Finally, several groups are evaluating the use of mAb-T-Ag fusion constructs that target the T-Ag to various DC surface molecules *in vivo* (e.g. C-type lectins such as DEC-205, DC-SIGN and Clec9A) or coating T-Ags with carbohydrates (e.g. Mannose) combined with DC maturating agents (e.g. TLR agonists) for more effective T-Ag uptake and DC activation [55•].

It is clear that there are multiple hurdles in mounting an effective anti-tumour T cell response, from limited T-Ag exposure of DCs and insufficient 'danger' signals within the tumour microenvironment, to insufficient $CD4^+$ T cell help for CTL generation. Further, the tumour microenvironment is often hostile to the emerging immune response, secreting suppressive immune mediators (e.g. TGF-β, IL-6, IL-10, IDO, VEGF and lactic acid) that can either directly down regulate DC T-Ag-processing and presentation (contributing to failed T cell immunity) or result in the recruitment and/or generation of suppressive cell types such as regulatory T cells or myeloid-derived suppressor cells that subvert the function of anti-tumour CTLs [18,56]. It is therefore likely that effective delivery of T-Ag and conditioning of DCs may not be sufficient to generate clinically effective anti-cancer immunity in the absence of strategies to overcome tumour-mediated immunosuppression. Thus, vaccine-based immunotherapy, which also targets the suppressive tumour microenvironment or suppressive networks induced by tumours, is more likely to succeed.

In this regard, there are several strategies being evaluated clinically to antagonise immunosuppressive networks present within cancer patients. These include depletion of regulatory T cells with IL-2-Diptheria toxin (ONTAK), cyclophosphamide to break regulatory networks or the use of anti-CTLA-4 mAb to 'take the brakes off' the system, in the hope of revealing the full potential of the cancer vaccine therapies in a suppressive cancer environment [57–59].

Conclusions

Recent studies in DC biology have shed new light on the complexity of how Ags are processed by DCs, the *in vivo* DC network, and the challenges faced with generating productive immune responses in the highly suppressive microenvironment of cancer patients. Even so, the immune system is immensely powerful as evidenced by its capacity to destroy kilograms of tissue in the setting of mismatched allo-transplantation and thus, if engaged and conditioned appropriately, should also be capable of killing tumour cells and rejecting solid cancers. As shown in this review, our knowledge of why DC-based therapies have failed is rapidly growing as are the number of strategies being employed to overcome or address these. We believe the future most likely lies with combination therapies that simultaneously deliver T-Ags and adjuvant to DCs, facilitate both their activation and efficient class I and II MHC/peptide presentation for T cell activation and condition the environment so as to overcome the immunosuppressive networks that have emerged during the *in vivo* evolution of the cancer.

Conflict of interest

EM is employed by CSL Limited, whose potential product was studied in the present work. None of the other authors have a financial interest to declare.

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